

Unravelling X-Inactivation through Time: Implications of X-Linked Disorders in Humans

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Abstract

X-Inactivation occurs early in embryogenesis, when one of the two chromosomes in each cell is subjected to specific modifications which lead to the formation of the transcriptional inactive heterochromatin known as Barr body. This mechanism was described 50 years ago by Mary Lyon. Scientists have been researching the phenomenon of X-inactivation ever since and multiple factors have been implicated in this multistep procedure. In our review we try to present how some of the mysteries of X-linked disorders in humans have been unraveled through time.

Keywords: X-chromosome inactivation; X-Inactivation centre; *XIST/Xist* gene; Skewed inactivation; X-linked disorders

Introduction

X-Inactivation: first findings

Sexual dimorphism in humans raised a number of intellectual queries and led the scientific community to decipher this phenomenon which still remains fascinating through time (Figure 1). The X-chromosome is 155Mb long and carries about 1,250 known genes. The Y chromosome being three times smaller has the lowest gene density known so far. In 1961, fifty years ago, Mary Lyon first hypothesized in a letter published in the Nature journal, that one of the X-chromosomes in each female somatic cell is randomly inactivated early in the embryonic development [1]. Although about 15-20% of the genes located mainly at the tips of the short and long arms of the X-chromosome (such as steroid sulfatase and Kallman syndrome genes) escape inactivation, the majority of genes on the inactive X (Xi) chromosome are silenced [2]. This results in dosage compensation of X-linked gene products between males and females [3-6].

The inactive X is clonally preserved in all subsequent cell divisions during embryogenesis. Therefore, females are considered mosaic for the X-linked genes. This statement was supported irrefutably by cytogenetic methods, biochemical evidence and animal experiments. Cytogenetically, the highly condensed inactive X-chromosome is observed as Barr body in all somatic cells with two or more X-chromosomes [7,8]. Biochemically the enzyme glucose-6-phosphate dehydrogenase (G6PD), has been used as a marker. It is known that the gene encoding G6PD lies on the X-chromosome and the enzyme is produced in equal quantities in both sexes. Should both X-chromosomes be active in heterozygous females an intermediate G6PD activity would be noticed. Conversely, biochemical tests have proved that female heterozygotes are potential genetic mosaics for the two common G6PD alleles (variants A and B) but not for the heterodimer. This results in the presence of two red cell populations, G6PD deficient cells and normal cells respectively [9,10]. Similarly, experiments on female mice or cats heterozygous for X-linked coat color genes exhibit different patches of pigmentation, while males do not [1].

Surprisingly enough, the inactive X-chromosome is reactivated in the female's germ line which results in the presence of active copies

from both X-chromosomes in fertilized egg cells [11,12]. Therefore, as X-Inactivation is reprogrammed in the fetus the selection of the X to be inactivated is not inherited in the next generation, and there is no mother to daughter transmission in humans.

Counting, choice and sensing

Over the years, X-Chromosome Inactivation (XCI) studies focused on patients with abnormal karyotypes where gain or loss of X-chromosomes has occurred [13]. XCI theories have been repeatedly revised due to novel molecular findings but three main key concepts of XCI remained constant [14,15]:

Counting: Where there is only one active X (Xa) chromosome and all the supernumerary X-chromosomes are inactive [3].

Choice: Where there is equality between the choice of the paternal or the maternal X-chromosome to be inactivated. The exception is when there are mutations or polymorphisms present on the X-Inactivation center [15].

Sensing/ Competence: This occurs when there is more than one X-chromosome in a cell and involving not only the XX recognition but the assessment of the X/autosome translocation and the ratio between X and autosomes [16].

Factors and mechanisms involved in counting and choice of the X to be silenced still remain under investigation. Several models have

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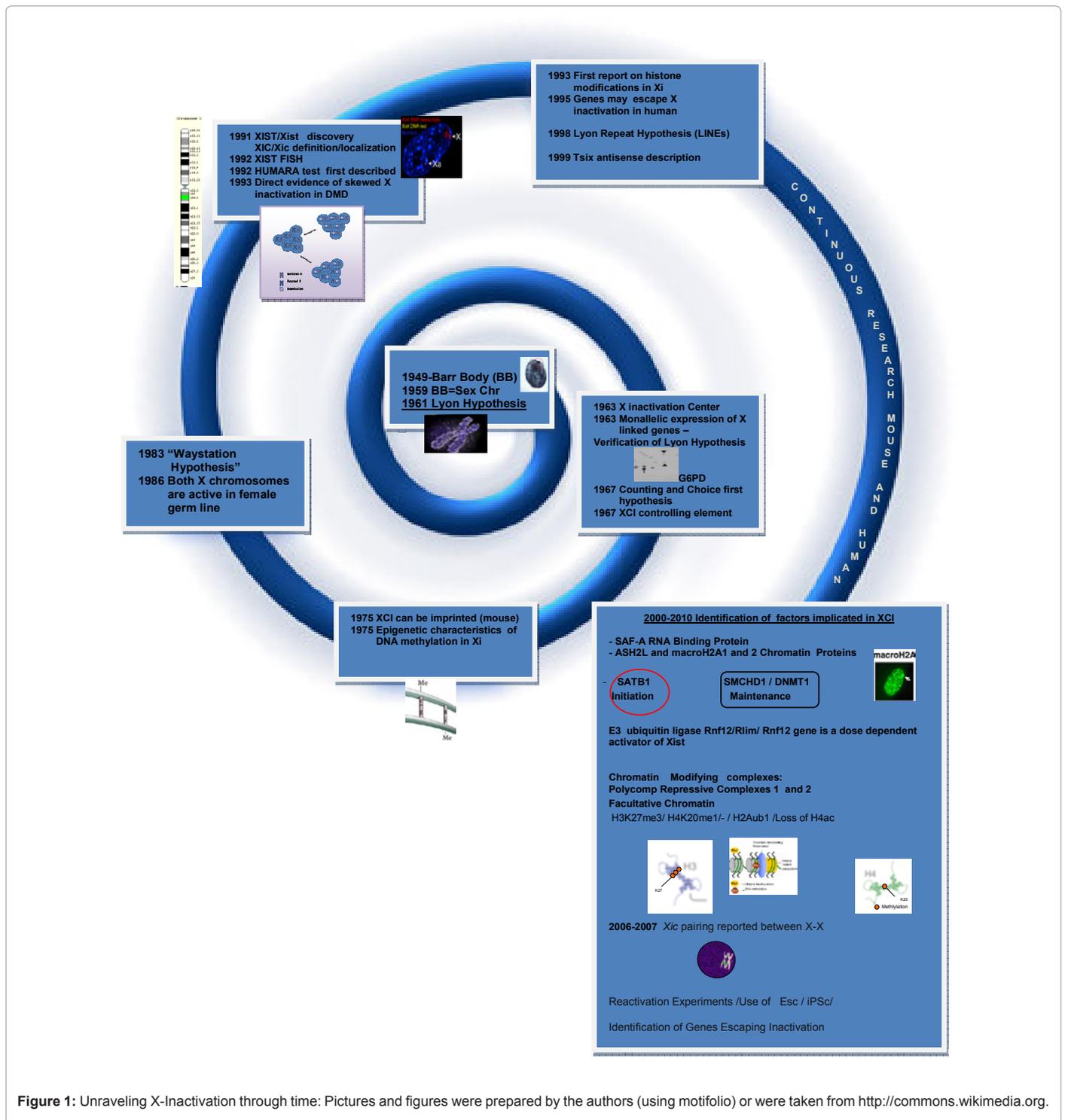


Figure 1: Unraveling X-Inactivation through time: Pictures and figures were prepared by the authors (using motifolio) or were taken from <http://commons.wikimedia.org>.

been proposed including: (1) The presence of blocking factors (BF) that protect the active X-chromosome (Xa) [17], (2) The effect of epigenetic markers on Xa and Xi before XCI [18], (3) The possible interaction between Xa and Xi [19] as well as (4) A stochastic model that assumes that both X-chromosomes can be inactivated with the probability that may be time dependent [17,20]. An evolution of the blocking factors (BF) model concerns a symmetry- breaking regulatory mechanism

where BF are self-assembled from diffusible DNA binding molecules. This procedure seems to be regulated by time in a way that only one BF is assembled, therefore breaking the binding symmetry of equivalent X-chromosomes [17,21].

X-Inactivation: understanding genetic events

During the last 50 years extended researches on X-Inactivation

have been performed and many genetic facts have been explained:

Male patients with 47, XXY (Klinefelter syndrome) have only one Barr body representing the inactivated X-chromosome.

X-inactivation results in equal blood protein product levels in males and females (gene compensation). The exception of the rule are the protein products of the genes located at the pseudoautosomal regions (PAR1 and PAR2) which are homology regions shared by both sex-chromosomes and found at each end of the X and Y chromosomes. It has been noticed that some of the genes escaping inactivation are located within the PAR regions.

All females are mosaic since one of the X-chromosomes is clonally inactivated and not expressed. This occurs in X-linked mutations (such as ocular albinism).

In carrier detection in X-linked disorders, cells expressing the normal allele have a selective advantage over the ones that express the mutant allele. This could explain the wide range of CPK levels in Duchenne Muscular Dystrophy (DMD) carriers or the distorted levels of the very long chain fatty acids measured in the carriers of the X-linked adrenoleukodystrophy [22].

Skewed (non random) X-inactivation in female heterozygotes explains the variability in manifestation of symptoms (manifesting heterozygote or carrier).

Studies in 46,Xr(X) females, where the *XIST* gene is not expressed on the ring X, have shown a severe phenotype attributed to functional disomy (biallelic expression for the genes which are not deleted) [22].

X-Chromosome inactivation (XCI) regulation control: the role of X-Inactivation center (XIC) and the *XIST* / *Xist* gene

XCI is controlled by the X-Inactivation Centre (XIC), located on the X-chromosome and has evolved exclusively in placental mammals. X-inactivation is an epigenetic phenomenon facilitated by differential methylation and is regulated by a gene located on the proximal long arm of the X-chromosome at the band q13 named *XIST* (X-Inactive Specific Transcript) [23-25]. The XIC consists of *XIST* and *cis*-regulatory elements and assures that all but one X-chromosome per diploid genome are inactivated [26,27]. Transcriptional silencing of one of the X-chromosomes in female cells is triggered by the molecular mechanism of XCI and the presence of the 17 Kb non-coding RNA of the *XIST/Xist* gene that binds *in-cis* and covers the inactive X (Xi) chromosome [28,29]. This multistep process involves initiation, spread of silencing and maintenance of the inactive state. These modifications ensure the stability of the inactive state during subsequent mitotic divisions. In a few families with marked skewed inactivation this has been attributed to an underlying mutation in *XIST* gene [30].

Elements influencing XCI

Once the choice of the X to be inactivated is made, initiation of chromosome-wide silencing takes place. So far, several factors have been known to influence XCI, the majority of which are *cis* and *trans* positive or negative regulators of *Xist* expressed by Xic [14,31,32]. The 17 kb non coding *XIST/Xist* RNA is expressed exclusively by the Xi [28,33]. Female cells carrying a 58 kb deletion including *Xist*, on one X-chromosome are able to initiate the XCI on the wild-type X-chromosome. Transgenesis studies with male mice embryonic stem cells carrying a *Xist*-containing YAC (up to 460 kb) showed that they

cannot initiate *Xist* during differentiation, neither from the transgene nor from the endogenous Xic [34,35]. Therefore, Xic-specific elements necessary to make cells competent for X-chromosome inactivation do not exist in these large DNA fragments. This implies that elements specific for *Xist* activation may be located not only near the gene but also, some distance away from the X-chromosome [14,36,37].

XIST is required for the initiation and establishment of genes silencing [38]. Maintenance of gene repression in differentiated cells however seems to be independent of *XIST* continued expression [32,39]. The preservation of X inactivated status implicates epigenetic mechanisms, such as chromatin condensation and facultative heterochromatin formation already recognized as the Barr body [32]. DNA and histone methylation, global histone hypoacetylation and Polycomb group complex (PcG complex) mediated chromatin modifications are important players in maintaining X-Inactivation [32]. *XIST* localization is important for XCI. SAF-A, a protein which is a part of the nuclear RNA-protein network organizing chromatin (nuclear scaffold), was characterized as a key factor for *XIST* in cis localization over the Xi [40,41]. On the other hand, aurora kinase B (AURKB) activity was shown to be related to *XIST* displacement from the inactive X-chromosome during chromosome condensation in mitosis in human cells [32]. *Xist* expression from transgenes that are integrated into various autosomes, as well as the ability of *Xist* to spread inactivation in X/autosome translocations, suggest that X-chromosome-specific sequences are not necessarily required for *Xist* localization. The comparison of the ability of translocations to spread and maintain XCI, aided Mary Lyon to discover the "repeat hypothesis" in 1998. According to this, repetitive elements (L1), a family of long scattered nuclear elements (LINEs) deriving from transposons, are over represented on the X-chromosome and determine *XIST* specificity [32,42-44]. The L1 density is extremely high around the *XIST* gene and very low around the genes that escape XCI [45]. Besides evidence relating to LINEs with XCI, over representation of additional repetitive elements such as the Long Terminal Repeat and the inverted repeat sequences on X-chromosome indicates that they may also be important players in XCI [32,46]. The hypothesis reported by Gartler and Riggs, suggested that similar elements also known as "way-stations" are dispersed along the X-chromosome and in this way facilitate the spreading of inactivation from the XIC [3]. On autosomes, these elements are absent or less frequent and restrict X-inactivation when X/autosome translocation exists [47]. When *Xist* is localized over Xi, RNA transcriptional activating histone modifications such as acetylation and H3 Lysine 4 methylation are absent. This is in consistence with the fact that no transcriptional activity is noticed in the *Xist* domain [32,48-51]. Similarly, absence of the transcription process, from the pericentric machinery suggests a possible equivalent chromatin reorganization coordinated by Polycomb Repressive Complexes 1 and 2 (PRC1, 2). PRC1 catalyses monoubiquitylation of histone H2A (H2Aub1) while PRC2 catalyses trimethylation of histone H3 at lysine 27 (H3K27me3), methylation of H4 at lysine 20 and deacetylation of H4 [32,52-57]. Engagement of PRC1 and PRC2 seems to precede gene silencing initiation and requires the presence of the *Xist* repeat A [32,48]. This conserved motif of RNA sequence located at the 5' end of *Xist* gene was shown to contain a 30 bp small core susceptible to interaction with proteins such as ASF splicing factor, EZH2, SUZ12 and PRC2 proteins [32,58]. SATB1, a special AT-rich sequence binding protein, acting as a silencing factor is thought to bind chromatin loops in a way that

involves Xist repeat and results in genes disposal to repression [59-61]. "Lock in" of XCI, is a stage where gene silencing is *Xist* independent and no changes of the X-Inactivation pattern are allowed [32]. Although a specific maintenance pathway has not as yet been defined, DNA cytosine-5-methyltransferase 1 (DNMT1) and structural maintenance of chromosomes hinge domain containing 1 (*Smchd1/SMCHD1*) seem to be necessary for "lock in" of XCI [32,62,63]. DNMT1 exerts the importance of DNA- promoter and CpG island methylation in XCI. Once gene silencing is "locked in" any reversal is considered impossible [32,64]. Even the use of ES cells and iPS cells in experiments aimed to reprogram cells, they revealed only partial reversal of the X-Inactivation status [36,65,66]. This high stability of gene silencing on Xi seems to originate from a specific chromatin constitution differing from other heterochromatins in which it is rich in *macroH2A* histone variant, SAF-A, SMCHD1 and Absent Small or Homeotic discs-like 2 (ASH2L) trithorax group (TrxG)protein[32,67]. In recent findings, it was suggested that a key role for the E3 ubiquitin ligase Rnf12/Rlim, a dose-dependent activator of *Xist*, is up-regulated during differentiation and found in abundance in female cells compared to male cells [68-71].

Despite the excess of novel data, there is still a great deal of controversy about the mechanisms involved in the whole process of XCI. In females with more than one X-chromosome, there is evidence that long range regulatory elements exist and are necessary for the specific up-regulation of *XIST*. After the up-regulation of the *XIST* gene and the start of the XCI on one X, the second allele of *XIST* is repressed on the active X-chromosome through DNA methylation of its promoter. Although not definitely proven, it has been suggested that the major *Xist* repressor is its antisense transcription unit, *tsix* which may play an important role in the cis-regulation of *Xist* [14,72-76].

Gene escaping X-Inactivation: the significance of 45, X studies

45, X patients have provided a helpful model for the study of DNA methylation. A comparative analysis with normal 46, XX females suggested the presence of imprinting genes that control social cognition [22]. Methylation studies for parent-of-origin specific methylation on the X-chromosome in Turner syndrome were negative, implying lack of such an imprinting on the human X-chromosome. Changes in methylation of CpG islands are correlated with the susceptibility to XCI [77,78]. This takes place in the promoter as well as in intergenetic CpG islands, however to a lesser extent. Genes which coexist on X and Y chromosome and seem to be homolog in some tissues require biallelic expression. These genes escape inactivation to ensure their expression. Therefore, it could be assumed that there are some X-chromosome regions where "way-stations" do not exist [47]. Sharp et al. [79], after a thorough DNA methylation analysis, on the active and inactive X-chromosomes, discovered that XCI is sensitive to changes in the methylation, not only in gene promoters but particularly at CpG islands which have increased methylation on the inactive X. However, the genes escaping XCI usually show lower levels of promoter methylation.

Their studies included measurements of methylation changes between two groups: one in 46,XX females and another one in patients with Turner syndrome (45,X0) [79].

Humans compared to mice showed a higher percentage of gene escaping inactivation. It is presumed that 10% of X-linked genes (approximately 31 novel genes) on the inactive X-chromosome seem to require biallelic expression from both the active and inactive X alleles.

This explains the previously unknown extent of heterogeneity among females and the severity of the phenotypic alterations observed in XO women. Variable degrees of escape have been reported to occur as tissue specific or even amongst cells of the same tissue [79].

X-Inactivation studies in X-linked disorders

Numerical, structural or locus specific abnormalities of the X-chromosome lead to X linked disorders of variable severity. Males carrying only one X-chromosome are more susceptible to X-linked mutations than females, making them more vulnerable to X-linked diseases and increasing the male mortality rate. In females, most X-linked genes are silenced due to X-inactivation and penetrance of symptoms seems to be related to X-inactivation profiles, especially if skewing has occurred in tissues amended by a specific X linked disease [80]. Many mental retardation (MR) syndromes are caused by a disturbance of genes involved in epigenetic mechanisms, particularly in X-linked MR and those related to sexual reproduction functions. About 10% of X-linked genes and only 3% of autosomal genes cause MR when mutated. In humans compared to rodents the X-linked genes, in contrast to the autosomal genes are highly expressed in the brain.

In a number of diseases, associated factors may alter the X-Inactivation pattern so that one of the two X will be selectively silent [81]. Non random XCI is characterized as primary, if it is caused by alterations concerning the molecular pathway of the X-Inactivation procedure [80,82]. Secondary X-inactivation selection is made either to sequester the negative effect of the mutated allele or as a result of increased cell proliferation rate induced by the mutation [43,80,82-86]. Even within females of the normal population various percentages of skewing have been observed [87]. However, a very small proportion may exhibit skewed inactivation, especially during the neonatal period [88]. Increased frequency of skewed X-inactivation was also reported in cells derived from patients with various types of cancer [89,90]. As this non random inactivation was observed in a wide range of neoplastic tissues it was postulated that it originates from the monoclonal origin of the neoplasia [81]. In 1993, Zneimer et al. [91] were the first to document extreme skewed X-inactivation in one of monozygotic female twins manifesting symptoms of Duchenne Muscular Dystrophy (DMD). In a recent review regarding the manifestation of symptoms in carriers of X-linked metabolic disorders, differences in clinical expression of heterozygous females was observed and probably resulted from cross-correction or cross-inducing mechanisms or by non random X-inactivation [87]. If non-random skewed X-inactivation occurs, an extensive phenotypic variability may be observed in carriers or female patients of X-linked disorders. Disorders with male lethality or reduced male viability such as RTT, IP and SCID are presented with skewed X-inactivation [87,92,93].

Rett syndrome

Rett syndrome is an X-linked dominant neurodevelopmental disorder often caused by mutations in the methyl-CpG binding protein type 2 gene (MECP2, OMIM # 300005) in chromosome Xq28 which is known to affect mainly females. The classical Rett symptoms are usually observed after the age of 6-18 months and consist of severe mental retardation, microcephaly, regression of motor skills, loss of speech ability and purposeful hand use [94,95]. The few male patients with Rett suffer from mental retardation, muscular hypotonia, progressive spasticity, seizures, poor speech and recurrent infections.

The duplication of the *MECP2* gene (Lubs syndrome) in Xq28, is genetically related to Rett syndrome and is one of the main causes of mental retardation in males, but rare in females. Substantial phenotypic variability could be attributed to the influence of X-chromosome inactivation (XCI) and the possible presence of some other modifier genes. It has been noted that the severity of the phenotype depends on the degree of skewed X-inactivation in Rett and Lubs syndromes. Knudsen et al. [96] found that mildly affected Rett cases were more skewed than the severely affected ones.

In mildly affected Rett patients, the active wild-type X may survive better and multiply faster compared to the cells with the active mutated X, thus improving the final clinical expression of the mutation [97]. Depending on the degree of the skewing, some individuals may present with a milder phenotype or “asymptomatic” such as healthy mothers of RTT patients who are carriers. Nielsen et al. [98], stated that the paternally inherited X-chromosome is preferentially inactivated in skewed Rett syndrome patients. Other studies have shown a higher frequency of skewed XCI in the blood of RTT patients than in normal individuals. This indicates that XCI differs from tissue to tissue, for example between blood cells, brain tissue, buccal epithelium or fibroblasts. Several reports showed that the pattern of XCI relates to the penetrance of RTT [98]. Research has been carried out to study the frequency of skewed X-inactivation but there are many conflicting opinions.

Incontinentia pigmenti

Incontinentia pigmenti (IP, OMIM 308310) is a rare X-linked

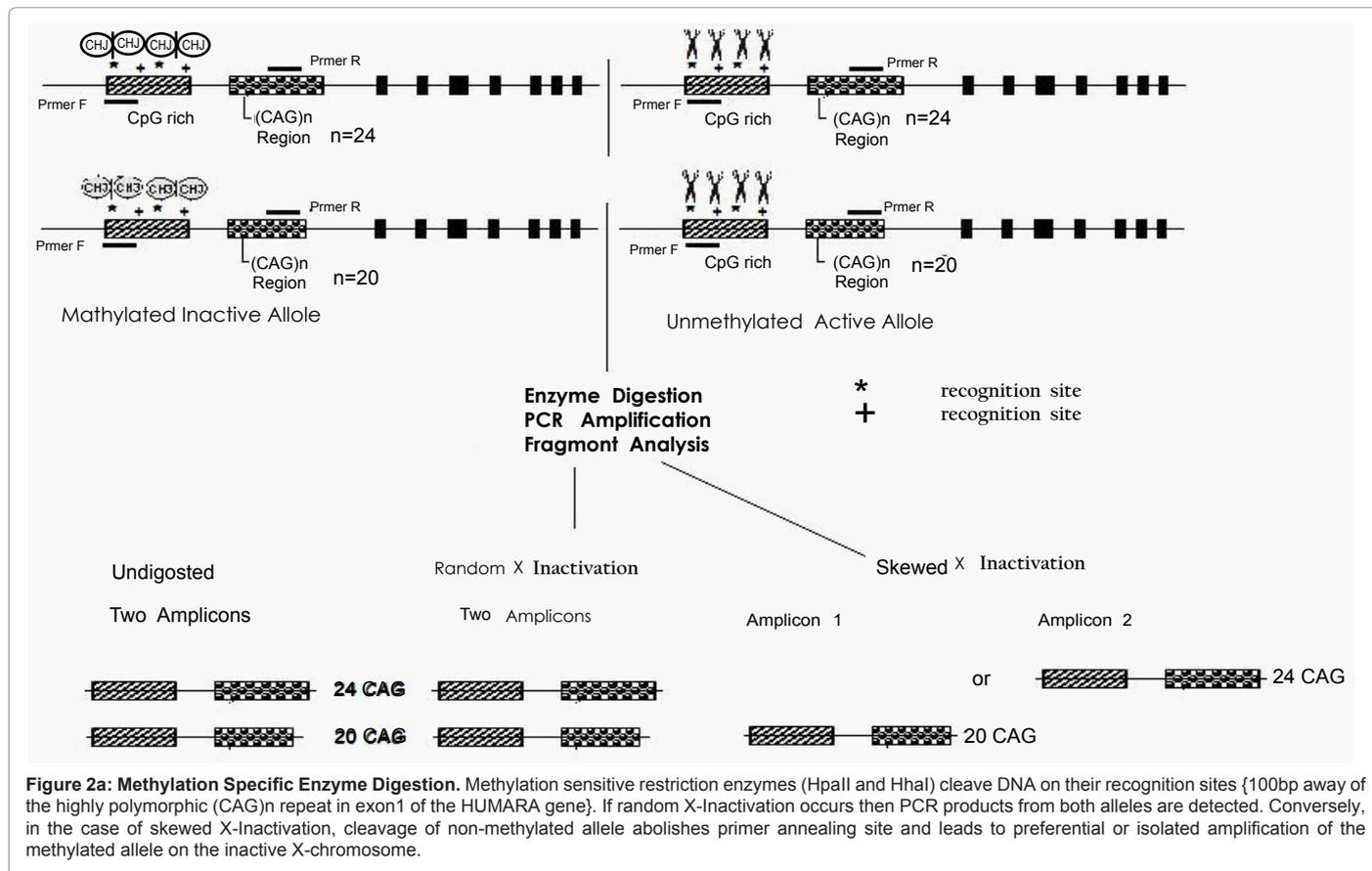
dominant genodermatosis, which occurs in approximately 1 in 50,000 newborns. It is characterized by cutaneous lesions and accompanied by neurologic, ocular, dental, hair, nail and musculoskeletal abnormalities [99].

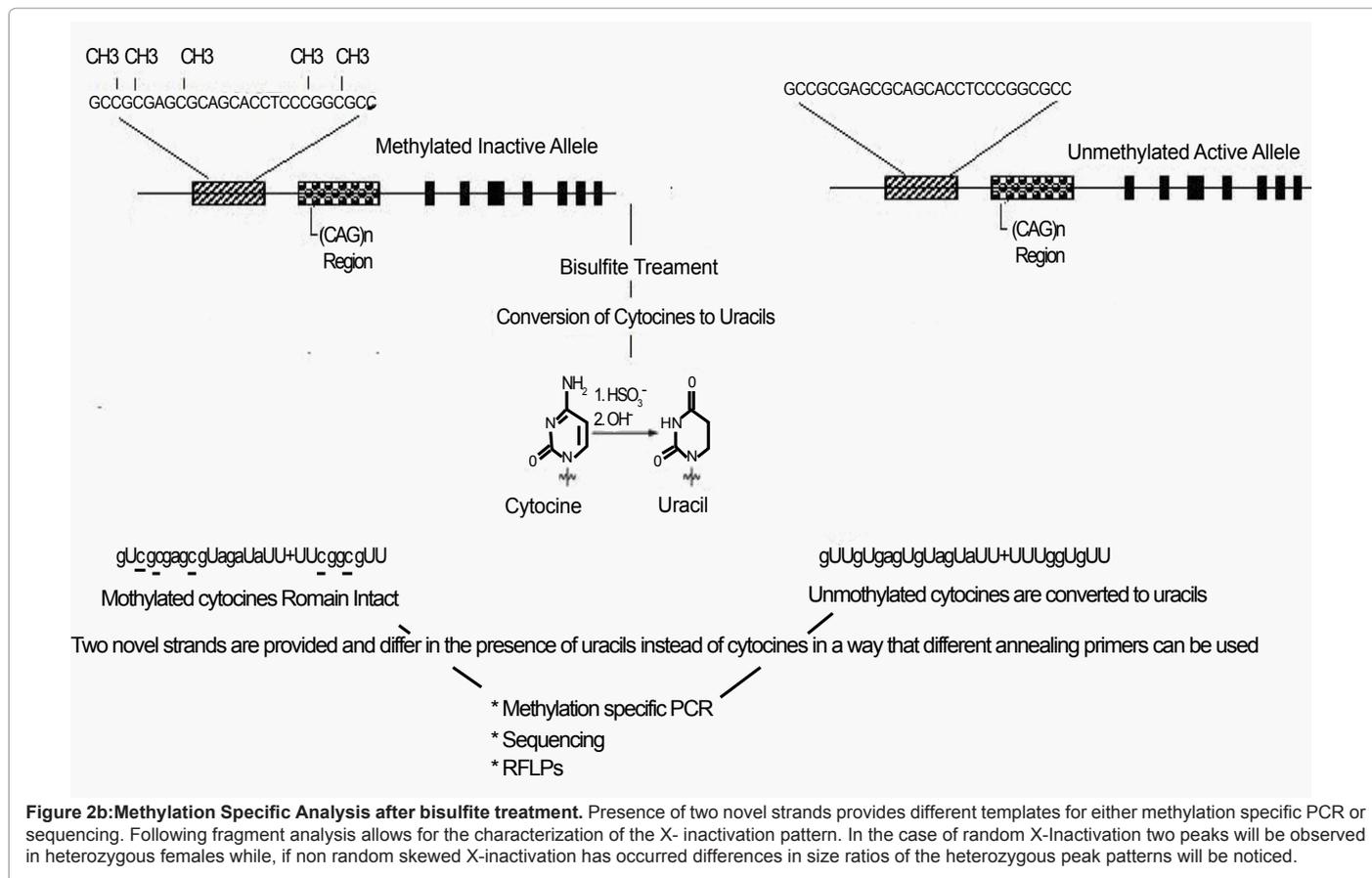
The molecular pathophysiology of the disease is attributed to mutations in the gene of nuclear factor kappa B (*NF-κB*) essential modulator (*NEMO*) currently known as *IKBKG* gene (Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma). The *IKBKG* gene is located on chromosome X, band q28. The vast majority of patients are females but there are some reports of the survival of male patients carrying the *IKBKG* mutation [100]. This is attributed to three mechanisms, either postzygotic somatic mosaicism, Klinefelter syndrome (47,XXY) or hypomorphic alleles (an allele that has reduced levels of gene activity) [101,102].

In males, IP is generally fatal antenatally. Females express variable phenotypic symptoms as a result of functional mosaicism due to lyonization. There is preferential inactivation of the mutant X-chromosome due to extremely apparent skewed X-inactivation. IP is an obvious example that females are mosaic as this can be seen by the cutaneous manifestations. The pigmentation abnormalities vary in extent and shape between heterozygotes females. This occurs because of the inactivation of the wild-type or the mutated X-chromosome in the precursor cells [103].

Severe Combined Immunodeficiency (SCID)

SCID is a rare inherited condition, either as an autosomal recessive or as an X-linked disorder. It consists of an absence of T-





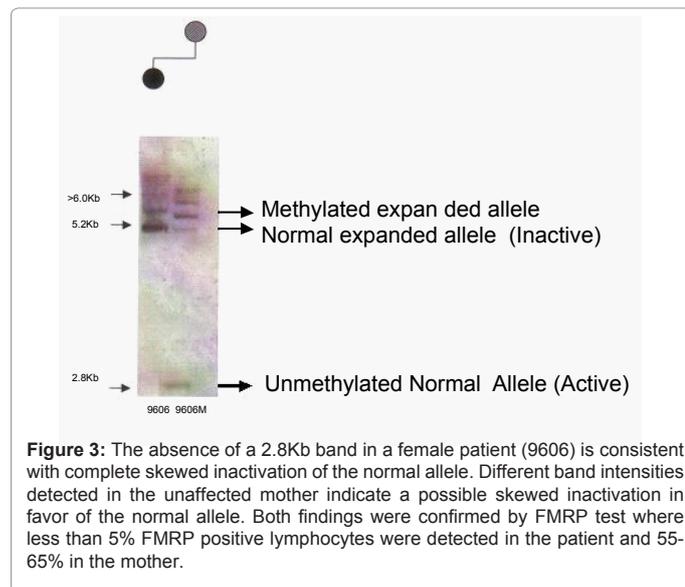
cells along with a functional defect in the B cells. The responsible gene for X-SCID (*IL2RG*) consists of eight exons which is 4.2 kb at Xq13. This gene encodes the gamma subunit that belongs to the receptors of several cytokines (interleukins 2,4,7,9 and 15), responsible for the development of T and NK cells and maturation of B cells. Various mutations in affected males have been reported so far [104]. Before the identification of the *IL2R*, methylation –sensitive restriction enzyme HpaII was performed to identify the randomness of X-inactivation. In X-linked pedigrees, obligate female carriers must have the defective X inactivated in all their lymphocytes. However, in their other tissues normal random X-inactivation has probably taken place. This is another example of skewed X-inactivation.

X-Inactivation studies: diagnostic approach and value

In the case of non random X-inactivation the skewed pattern concerns the preferential inactivation of the paternal or the maternal chromosome respectively and may serve as a diagnostic –prognostic tool. Most methods performed to allow characterization of X-chromosome inactivation (XCI) status are based on the fact that transcriptional inactivation is established by the extensive allelic methylation of CpG islands [83]. The most common region studied, to assess differential methylation between active and inactive X-chromosomes, is the human androgen receptor gene (HUMARA). Exon 1, contains nine CpG regions where primer sequences can be designed. High heterozygosity rates (>98%) of CAG polymorphic region in HUMARA exon 1 helps identification of maternally and paternally derived alleles. HUMARA’s promoter methylation status coincides with that of the

enzymes recognition sites and is strongly correlated to X-inactivation. HUMARA assay allows for the distinction between maternal and paternal alleles as well as characterization of their methylation status [83]. Methylation patterns were studied by methylation specific enzyme digestion followed by Southern blot analysis or PCR (Figure 2a). With the new molecular technologies a more specific and accurate procedure has been developed and involves a chemical reaction where bisulfite treatment allows for the conversion of unmethylated cytosine residues to uracil. Sodium bisulfite modification alters cytosines of CG dinucleotides on the active chromosome, while methylcytosines in the inactive X-chromosome remain unaffected. Specific primer sets are designed to anneal on either cytosine or uracil strands and amplify PCR products deriving from methylated HUMARA alleles on the inactive X-chromosome or unmethylated alleles on the active X-chromosome respectively [105] (Figure 2b). Recently, more specific studies included the use of methylation- specific microarrays or methylation specific Multiplex Ligation Probes Analysis (MLPA) performed after genomic DNA bisulfite treatment [105].

Evaluation of the X-inactivation status- especially if a skewed profile is suspected- may provide a valuable prognostic tool in female patients [88]. In fragile- X where the diagnosis can be made through Southern Blot analysis the use of additional methylation specific enzymes (EagI), allowed for skewed X-inactivation to be revealed. In this way the complete loss of FMR-1 activity in a severely MR female patient and the lack of any symptoms in carriers of a methylated expanded allele were explained [106] (Figure 3). Similarly, in IP when the common



IKBKG gene deletion is excluded, skewed X-inactivation (non-random selective inactivation of the mutant X-chromosome in most of the cells) is observed. The complete digestion of one allele is indicative for skewed X-inactivation which reduces diagnostic confusion [103].

Methylation specific enzyme digestion

Methylation sensitive restriction enzymes (*HpaII* and *HhaI*) cleave DNA on their recognition sites (100 bp away of the highly polymorphic (CAG)_n repeat in exon1 of the *HUMARA* gene). If random X-inactivation occurs then PCR products from both alleles are detected. Conversely, in the case of skewed X-inactivation, cleavage of non-methylated allele abolishes primer annealing site and leads to preferential or isolated amplification of the methylated allele on the inactive X-chromosome (Figure 2a,b).

Recovery of DNA during bisulfite modification extends to 80-90%. Presence of two novel strands provides different templates for either methylation specific PCR or sequencing. Following fragment analysis allows for the characterization of the X-inactivation pattern. In the case of random X-inactivation two peaks will be observed in heterozygous females while, if non random skewed X-inactivation has occurred differences in size of the heterozygous peak patterns will be noticed.

Concluding Remarks and Future Perspectives

The hypothesis that genetically identical chromosomes, active and inactive, could coexist in the same nucleus was at first disbelieved by many geneticists. Researchers are still continuing studies on the epigenetic mechanisms to unravel the mysteries of X-Inactivation. Although there are many differences between mice and human X-Inactivation, such as the sequence of *Xic* and phenotypic outcomes of X-chromosome abnormalities, mouse experiments have helped in the understanding of the epigenetic mechanisms of XCI.

Ongoing research brings novel insights regarding the involvement of additional genetic key factors regulating XCI multistep procedure. Future studies should focus on deciphering why *XIST* expression is restricted in female cells and moreover only on the *Xi* and not on the *Xa* allele [71]. Characterization of *Xi* loci where *Xist* non coding

RNA binds in order to allow facultative heterochromatin formation and identification of possible molecular pathways related to gene silencing, will also help our better understanding of XCI [32]. In addition, unravelling the exact mechanisms underlying non random X-Inactivation is expected to allow better evaluation and most proper genetic counselling of female carriers of serious X linked conditions.

Skewed XCI, a key factor in the manifestation of symptoms in heterozygous females, should be further investigated to address whether this complex trait results from stochastic events or selective favouritism. In the case of tumors skewed inactivation may be the cause of tumor susceptibility. It is however possible that both non random inactivation and elevated cancer risk result from common or even independent events [90]. Most of the diseases exhibiting skewed inactivation include mental-retardation or immune-deficiency, whether this non random X-Inactivation is observed only in this type of X-linked mutation or concerns all X-linked genes, remains to be seen [107]. So far, there is only one report of a family where more than one individual shared the same skewed pattern, possibly due to a mutation of the *XIST* gene [82]. There is no other evidence that skewed X-Inactivation is transmitted [108]. Unravelling the exact mechanisms underlying non random X-Inactivation is expected to allow better evaluation and proper genetic counselling of female carriers of serious X linked conditions.

Methylation of CpG islands is the major mechanism serving to "lock in" X-Inactivation. Incomplete this or hypo-methylation may be a factor enabling reversal of X-Inactivation in certain types of cells such as the placenta cells [109]. Human iPSCs are still extensively studied to allow evaluation of their genomic and epigenomic integrity as well as their potential therapeutic utility. However the results from experiments in mice are most promising as they suggest that epigenetic reprogramming as well as treatment of genetic disorders with iPSCs is feasible [110]. Recent studies concerning methylation profiling in hESCs, (hematopoietic embryonic Stem Cells) report that during hematopoietic differentiation reversal of the *HUMARA* methylation can occur in differentiated progenitors, both in mice and human models [111]. hESCs are important in developmental studies as well as for future clinical and therapeutic approaches of disorders that involve mutations in X-linked genes subjected to skewed X-Inactivation such as muscular dystrophy and Rett. Restoring *MECP2* function in the brain of mice was shown to reverse the symptoms suggesting that if an intact copy of *MECP2* was inactive, selective reactivation could serve as a potential cure.

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