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Germ-line epimutaions and human cancer

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[Abstract] Epimutations are errors in the normal process of epigenetic regulation which can result in aberrant transcriptional silencing of a normally active gene or reactivation of a normally silent gene. Epimutations are generally considered to be somatic events and to be confined in affected tissues. However, recent studies of patients with hereditary nonpolyposis colorectal cancer (HNPCC) have showed that allele-specific hypermethylation of CpG islands in the promoter region of the MLH1 gene, one of the causes of the tumor, existed in all the tissues examined. In addition, germ-line epimutations of other tumor suppressor genes (TSGs), such as MSH2 and BRCA1, have also been reported, demonstrating that epimutations might arise in the germ-line (during gametogenesis or early embryonic development). The role of germ-line epimutations might be as important as germ-line mutations in human disease. We reviewed the update on germ-line epimutations of TSGs including the possible mechanisms underlying germ-line epimutations, the possibility of transgenerational inheritance, and their impact on our understanding of human disease.

Key words: germ-line epimutation, tumor suppressor gene, hypermethylation, MLH1

Epig enetic study is about the changes of heritable gene expression which are not related to DNA sequence changes.[1] Epigenetic mechanisms play an important role in tissue-specific gene expression during differentiation, resulting in developmental plasticity. Epigenetic mechanisms mainly include DNA methylation, histone acetylation, methylation-induced chromatin structure alteration, and posttranscriptional regulation of non-coding RNAs. Among them, DNA methylation is the most extensively studied epigenetic modification, which transfers genetic information of non-DNA sequence in the process of mitosis or meiosis. The genetic information regulates gene expression in a time and space manner during embryonic development, and maintains the differentiation of a variety phenotypes of cells in individuals.[2-4]

In mammalian cells, DNA methylation occurs mainly at the C5 position of CpG dinucleotides, that is, 5'-m5CpG-3'. Human genome includes 28 millions of CpG dinucleotides, 7% exist in CpG islands.[5] CpG islands are rich in CpG dinucleotides with a length of 200–500 bp in genome. In human, nearly 70% of gene promoters contain CpG islands.[6] In normal cells, most CpG dinucleotides (about 70%) are methylated, unmethylated CpG usually exist in the CpG islands of gene promoters.[7]

Epimutations are errors in epigenetic regulation, which result in aberrant transcriptional silencing of a normally active gene or reactivation of a normally silent gene.[8] In 1987, Holliday proposed that epimutations could cause diseases.[9] Many studies in recent years supplied more and more evidences to support this hypothesis. For example, hypermethylation of CpG islands in the promoter region of a certain gene leads to gene silencing, the mechanism may be the methylation of CpG islands suppressed the binding between transcriptional factor and DNA or between methylation-binding protein and DNA.[10] By inhibiting the transcription of involved allele genes, epimutantions can efficiently reduce gene expression, even completely abolish, resulting in the same effect of gene sequence mutation, finally cause phenotypic changes in cells or individuals, even lead to diseases.

Previously, epimutations are generally considered to be somatic events, such as the epimutations of tumor suppressor genes (TSGs) that widely exist in tumor tissues during tumor progression. However, recent studies showed that epimutations not only existed in somatic cells, but arised in the germ line (gametogenesis or early embryonic development), and maintained in the whole process of individual development, involved in all tissues and transmitted to offspring. That is called germ-line epimutatons. Previous studies on germ-line epimutations were limited to plants and animals, until recent years, germ-line epimutations in human have great progress. This paper reviews recent proceedings in germ-line epimutations. We mainly discuss the germ-line epimutation of TSGs in human.

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Germ-line epimutations in eukaryotes

Significant progress has been made in investigating germ-line epimutations in plants. P site in maize,[11] SUPERMAN gene in *Arabidopsis thaliama*[12] and Lcyc gene in toadflax[13] all have aberrant methylations, leading to lost of gene expression and changes of plant phenotype. In addition, those epimutations can be transmitted to offspring.

The germ cells and somatic cells in plants do not completely separate in early embryonic development, thus, it is not strange that the epimutations could be transmitted to offspring through sexual reproduction or nutrition reproduction. However, the same phenomenon also happens in mammals.

A part of murine genes show expression diversity which can not be explained by Mendel's laws. These genes are called metastable epiallels. The most famous ones are A^{vy} (agouti viable yellow) gene and $Axin^{Fu}$ (axin fused) gene.[14-16] Their expression depends on the methylation of long terminal repeat (LTP) in an intracsternal-A particle (IAP) which is near or inside the gene. Hypomethylation of upstream IAP of A^{vy} gene causes agouti gene expression, with which the coat color of mouse is yellow; while hypermethylation leads to agouti gene silencing, and the coat color is gray. Hence, the mice carrying A^{vy} gene (A^{vy}/A) show mosaic phenomenon in coat color, ranging from gray (like wild type) to sallow mosaic and completely yellow.[15] IAP is included in the sixth intron of $Axin^{Fu}$ gene, hypomethylation of IAP results in increasing aberrant transcription of $Axin^{Fu}$ gene, leading to abnormal tail development in mice. The malformations of the tail are attributed to methylation of IAP.[16]

Germ-line epimutations not only happen in animals and plants but also exist in human imprinting gene diseases. Prader-Willi syndromes (PWS) and Angelman syndromes (AS) are genetic disorders caused by functional deficiency of a group of imprinted genes in 15q11-q13. In the patients with imprinting gene defects, only a few have microdeletions in imprinting center. In the patients with imprinting gene defects without sequence deletion, epimutations in imprinting center are the direct cause of diseases. In PWS patients, all involved genes are inherited from grandmother, whereas, in AS patients, one part of the involved genes is inherited from maternal grandfather and the other part from maternal grandmother. According to experimental data, researchers suggested that the epimutations occur in the period of germ cell formation, and may be due to incompletely deletion of epigenetic marks in maternal chromosome during the sperm formation (PWS patients), which belongs to germ line alteration and would cause serious clinical disease phenotype.[17]

Whether the changes also happen in non-imprinting genes? Researches on TSGs give a positive answer. Germ-line epimutations not only explain the genetic traits but also reveal the pathogeny of some diseases.

Research breakthrough of germ-line epimutations in tumor suppressor genes

Comparing with normal cells, tumor cells exhibit decreased genome-wide methylation status (reduced metylation of DNA repeat sequences, gene coding and intron regions, and some oncogenes) and increased methylation of the CpG islands in the promoters of TSGs.[18,19] The methylation of CpG islands was first found in Rb gene promoter in retinoblastoma, then found in other TSGs in other tumors, such as cell cycle-related gene P16INK4a,[20] DNA damage repair-related genes hMLH1[21] and BRCA1,[18] and tumor suppressor gene CDH1.[22] Recent researches showed that abnormal DNA methylation not only happened in tumor cells but also came from all germ layers of non-tumor cells, with a germ-line origin.

Germ-line epimutations of MLH1 gene

Hereditary nonpolyposis colorectal carcinoma (HNPCC), a somatic chromosome dominant genetic disease,[23] is related to silencing of mismatch repair genes (MMR), MLH1 or MLH2.[23] In 2002, Gazzoli *et al.*[24] detected DNA hypermethylation of one allele in the CpG island in the promoter region of MLH1 gene in DNA isolated from blood in one of 14 suspected HNPCC patients. Further analysis confirmed that the unmethylated MLH1 allele was eliminated by loss of heterozygosity (LOH) and the methylated allele was retained. No germ-line sequence mutation in HNPCC-related gene was detected in this patient. Thus, Gazzoli *et al.*[24] suggested that the methylation and silencing of MLH1 gene was a germ-line alteration, which was related to tumorigenesis. These results suggest a novel mode of tumorigenesis, which confirms to the two-hit hypothesis, that is, germ-line methylation of TSGs is the first hit and LOH causes the tumorigenesis finally.

In 2004, Suter *et al.*[25] reported two HNPCC patients with soma-wide, allele-specific and mosaic hypermethylation of MLH1 gene. In both patients, promoter methylation of MLH1 was detected in normal somatic tissues with distinct embryonic origins. These normal tissues include buccal mucosa originated from the endoderm, peripheral blood originated from the mesoderm, and hair follicles originated from ectoderm. These results imply that it occurs as a germ-line mutation. Germ-line epimutations can cause the same disease phenotype as that caused by gene sequence mutation. Therefore, the susceptibility to specific diseases of the individuals with germ-line epimutations is increased.

Later, other reports confirm these findings, and improve the understanding of germ-line methylation. In 2005, Hithins *et al.*[26] found that among 160 probands of HNPCC pedigree, one man had soma-wide monoallelc hypermethylaton of MLH1 promoter. Moreover, only unmethylated MLH1 gene was expressed in peripheral blood of the patient, indicating that the methylation of MLH1 gene caused transcriptional silence. In addition, no aberrant methylation of MLH1 gene was detected in 300 healthy controls, indicating this molecular defect was disease-associated. Two years later, in 2007, Hitchins *et al.*[27] detected allele methylation in MLH1 promoter in two of 24 patients with colorectal cancer or endometrial cancer, younger than 50 years; they found that the molecular defect was restricted to a single allele and was presented in somatic tissues derived from all three embryonic germ cell layers, and the methylated allele gene was transcriptionally silenced. More importantly, they first reported the

transmission of MLH1 epimutations between generations.

In 2008, Morak *et al*.[28] expanded the sample size, detected MLH1 promoter methylation in 12 of 94 HNPCC patients, one of whom transmitted the methylated MLH1 gene to her son. Therefore, in the population with suspicion of HNPCC as well as deletion of MSI-H and MLH1 expression, while without MLH1 germ-line sequence mutation, the detection rate of MLH1 promoter methylatation reached to 13%, some patients showed a mosaic methylation.

At present, there are 7 papers reported 25 patients with MLH1 promoter germ-line methylaton.[24-30] These researches show that similar to germ-line sequence mutation, MLH1 germ-line epimutations lead to MLH1 and MSI-H deficiency, causing suspected HNPCC. However, different from sequence mutation, epimutation can reverse to normal status, therefore, it demonstrates a high degree of variability, making germ-line mutation showed mosaic and week inherited ability.

Germ-line epimutations of other tumor suppressor genes

In 2006, Chan *et al*.[31] detected germ-line allele-specific and mosaic hypermethylation of MSH2 gene, without evidence of DNA mismatch repair gene mutation in a HNPCC family, with inheritance in three successive generations. In 2008, Snell *et al*.[32] found mosaic methylation in the BRCA1 promoter region in the peripheral blood of three patients with familial breast cancer. Buccal mucosa DNA from one of these patients displayed low level of BRCA1 gene metylation. These results demonstrate that in some breast cancer patients, low-level promoter methylation of BRCA1 occurs in normal tissues and is associated with the tumorigenesis. Additionally, in 2008, Romero-Gimenez *et al*.[33] detected germ-line methylation of APC in familial adenomatous polyposis (FAP) patients, while found no germ-line epimutations.

These studies demonstrate that germ-line epimutations of some TSGs play a role of first hit in two-hit hypothesis, comprise the causes of some inherited tumors. The study of germ-line epimutations of TSGs has just begun. Whether this mechanism occurs in other tumors and the proportion in tumorigenesis is unknown. Since DNA methylation has been considered to be a reversible change, different from sequence mutation, it can become a novel approach for clinical cancer therapy.

Mechanisms of germ-line epimutations

Epigenetic gene silencing is a complex process, includes many different factors, involves interactions among proteins, DNA and RNA, and may happen in all phases of mammal development. In the past 30 years, studies showed that the establishment of individual epigenetic markers undergone two important periods, that is, gametogenesis and early embryonic development. In these two periods, epigenetic markers reprogram in cells via eliminating original epigenetic markers and reestablishing new epigenetic markers.[34] Germ-line epimutations may arise from the errors in reprogramming. In the process of reprogramming, the methylations of some sites are maintained or established mistakenly, which may happen randomly or be affected by environm ent. This phenomenon is called primary germ-line epimutations. On the other hand, germ-line methylations can be regulated by in *cis*-acting element or *trans*-acting element, which is called secondary germ-line epimutations.[35]

At present, possible relationship between *in cis*-acting element and MLH1 germ-line epimutations can be excluded because not all individuals with same haplotype carry MLH1 germ-line epimutations.[27] Although it can not completely exclude the effect of *trans*-acting element regulation, no evidence has been found. Thus, MLH1 epimutations are likely to be primary germ-line epimutations that arise randomly or affected by environment, causing aberrant methylation in early embryonic development. These changes happen in MLH1 gene locus primarily, that is, aberrant hypermethylation of CpG islands in MLH1 promoter, lead to decreased protein expression of MLH1 in somatic cells and induce tumorigenesis.

Animal experiments indicate that environment factors play significant roles in the formation of germ-line epimutations. These factors may directly lead to the formation of germ-line epimutations. During gonadal development of early embryo in rats, exposure to the environmental endocrine disruptors vinclozolin and methoxychlor induces an adult phenotype in the F1 generation of decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility. These effects can be transferred through the male germ line to males of subsequent generations examined, that is, F1 to F4. Abnormal DNA methylation of genes, including lysophospholipase (LPLase) and cytosine-inducible SH2 protein, is detected in the involved male rats. Thus, environmental endocrine disruptors may affect reprogramming of germ-line methylations in early gonadal development and epigenetic of germ cells, promote a disease etiology in rat.[36] Except for environment toxins, the amount of metyl donor in mid-gestation diet of female mice also affects the methylation status of A^{vy} gene.[37] In addition, environment factor not only affects gamete zygote formation and early embryonic development but also acts on the period after birth. In the first week postpartum, the activity of pup licking/grooming of mothers to infants affects the promoter methylation and expression of estrogen receptor α (Erα in the medial preoptic area (MPOA) as well as activity of estrogen in infant rats, therefore, affects the behavior of female offspring as mothers, maintains the epigenetic transmission in subsequent generations. [38]

Some potential gene functions comprise the main reasons of secondary germ-line epimutations, including *in cis*-acting and *trans*-acting regulation. In imprinting gene disease Beckwith-Wiedemann syndrome (BWS), some patients have maternal 1.4–1.8 kb microdeletion in imprinting center region, which is associated with the methylation status of maternal imprinting genes, and the mechanism might be that microdeletion decreases the binding sites of CTCF protein.[39] In most chronic lymphocytic leukemia (CLL) patients, down-regulation of tumor suppressor gene DAPK and abnormal methylation of DAPK1 promoter are detected. Furthermore, a SNP site (c.1-6531A>G) of DAPK1 gene is related to DAPK1 down-regulation. These results suggest that this SNP site enhances the DNA-binding

affinity of HOXB7 protein as a mechanism predisposing to CLL, while HOXB7 inhibits DAPK1 expression, and the down-regulaton of DAPK1 via histone modification finally causes abnormal methylation.[40] Epimutation analysis of another mismatch repair gene MSH2 shows its relationship to certain genotype.[31] Individuals of this family with the same haplotype as proband carry this epimutation, hence, the gene silencing may be triggered by certain *in cis*-acting element instead of the function of primary germ-line epimutations.[41-43]

Recent study showed that uncoding RNA play an important role in the formation of heterochromatin, as well as mediating and maintaining epigenetic silencing through targeted binding to special region of gene sequence. This function attributed to the *trans*-acting in the formation of epimutations. In the differentiated mice embryonic stem cells, inducing antisense transcription of HBA2 gene causes abnormal methylation of CpG islands and silencing of HBA2 gene expression.[44] In leukemia cells, antisense RNA expression of P15 gene is higher than that in normal cells. Introducing antisense sequence of P15 to differentiated mice embryonic stem cells results in changes of histone acetylation and methylation status, follows by hypermethylation in P15 promoter region, causes a consistence inhibition of P15 sense expression and attributes to mechanism of leukemia genesis.[45]

Inheritance of germ-line epimutitions

Animal experiments proved that germ-line epimutitions could be transmitted to offspring. The critical example is mice. The methylation statuses of A^{vy} and $Axin^{Fu}$ genes in parental mice affect the methylation statuses in offspring (hypermethylation in parental tend to generate hypermethylation in offspring). This inheritance comes from either mother or father.[14-16] The content of folic acid in food and the germ line influence this inheritance,[37] showing a difference from critical mutation in gene sequence, is a non-Mendel's Law manner.

In the study of MLH1 germ-line epimutitions, evidences about inheritance of germ-line epimutations have been found.[27,28] Hitchins *et al.*[27] reported that the hypermethylated allele was maternally transmitted, MLH1 germ-line methylation was found in one of Patient A's sons (family member Ⅱ 6-a) and the maternal allelic gene was silenced, but his sperm had no MLH1 methylation, furthermore, his sperm showed reactivation of the maternally derived MLH1 allele. In addition, Patient A's sister and the other two sons with the same haplotype all did not carry MLH1 germ-line epimutation. Patient B's mother and her two sons carried the same haplotype, but had no MLH1 methylaton. Morak *et al.*[28] analyzed the methylation status of four HNPCC patients with germ-line epimutations. Among them, one showed transmission of abnormal methylation of maternal allele to offspring, two showed new methylation of maternal allele, and one showed no transmission of methylation to his son.

Above researches indicate that germ-line epimutations have the capacity of inheritance, however, this capacity is week, which is in consistence with non-Mendel's law in animal study, that is, epimutations could reverse to normal wild phenotype in some situations. Hitchins *et al.*[27] found that the mother transmitted the involved allele gene to multi-offspring, but the epimutation only maintained in one offspring. Those who lost the epimutation may due to the epimutation either happened in maternal germ cells or had been erased in early embryonic development. The mutation reversal also can explain the germ-line epimutations in individuals who present different degrees of cell mosaic. Since the germ cells are not assessed, the inheritance of germ-line epimutations has not been fully recognized. Some individuals may transmit germ-line methylation to offspring directly, or the epimutations are erased in early embryonic development, some *trans*-acting elements reestablish these epimutations. However, which *trans*-acting elements cause the epimutation is unknown.[8] In the other hand, different involved genes display different genetic modes. With deep understanding of epimutations, this genetic mode will become clearer.

Summary and prospect

Finding human TSG germ-line epimuatations gives an important significance. It not only puts forward a new mechanism of tumorigenesis but also indicates that germ-line epimutations may relate to wide range of human diseases. The research of germ-line epimutations is particularly important for those patients who suffer from genetic diseases, but with no mutation of related gene being screened. For example, the screen targets of MLH1 germ-line epimutations are those who are young, with no family history, and with MSI tumors and MLH1 expression deletion. However, if a particular disease or syndrome is not associated with a particular genetic mutation, searching for possible germ-line epimutations in a wide range of genome is relatively difficult. Relative weak capacity of inheritance of germ-line epimutations and frequent reversibility form a mosaic and non-Mendel's law genetic model, making genetic map being relatively difficult to be drawn. On the other hand, germ-line epimutations show mosaic and non-Mendelian characteristics, which may explain the phenotypic variability and changes in the genetic penetrance in some complex diseases, thereby, give a novel way to study the etiology of complex diseases. Some diseases previously known as complex multi-gene genetic diseases may be explained easily by single gene epimutiation.[46] Whereas, there are still many questions need to be answered, and it is not difficult to imagine that similar studies will develop in other tumors, even in genetic diseases other than tumor. With further researches in germ-line epimutations, it will be possible to expand the genetic basis of diseases and provide a new way for clinical diagnosis and therapy.

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