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Epigenetic aspects of MDS and its molecular targeted therapy

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Abstract The term "epigenetics" refers to clonally inherited stable variability in gene expression without underlying genetic changes. There are two well-known molecular mechanisms for epigenetic information: DNA methylation and histone modifications. Epigenetic changes have been recognized in the past decade as critical factors for physiological phenomena such as embryogenesis and the differentiation of normal cells. There is recent interest regarding the involvement of aberrant DNA methylation and histone modifications in mediating altered physiology in cancer. MDS is characterized by epigenetic changes, mutations in epigenetic regulators, and response to DNA methylation inhibitors, suggesting that epigenetic changes are unique features of MDS patients. In this article, recent progress in the understanding of MDS epigenetics and epigenetics-based therapies is reviewed.

Keywords Epigenetics · DNA methylation · Histone modification · MDS · Mutation

Epigenetics

Epigenetic changes, heritable through mitosis, lead to variability in gene expression without affecting the genetic sequence. These epigenetic processes play an important role in the early stages of natural development, from embryogenesis to the determination of cellular fate for commitment to their lineage [1]. Epigenetics process can also result in biological diversity and phenotypic variation. Given that cancers are considered to have variable phenotypes, it has been appreciated that dysregulated epigenetic mechanisms can be a fundamental mechanism in cancer, and targeted therapy for these processes is of clinical interest [2, 3]. Besides DNA methylation, which is the most studied epigenetic mechanism in cancer, post-translational histone modifications have also been found to mediate epigenetics (Fig. 1).

DNA methylation is the addition of a methyl group to cytosines at the 5′ position of a CpG dinucleotide by a covalent modification which results in the formation of 5-methylcytosine (5mC), a base that changes the interactions between protein(s) and DNA. In mammalian cells, DNA methylation is a replication-dependent reaction catalyzed by DNA methyltransferases (DNMTs) which are present at the replication fork during the S-phase [4]. CpG dinucleotides are typically rare and scattered throughout the genome and are fully methylated. However, DNA methylation also involves CpG-rich regions called "CpG islands" (CGIs) [5]. These have been shown to be present in approximately half of the human gene promoters. CGI methylation is associated with absent transcription from the involved promoter, such as that shown in the inactive X chromosome in women [6] and in imprinting [7, 8]. The mechanism whereby CGI methylation suppresses gene transcription has been partially elucidated in vitro [9]. DNA methylation leads to silencing directly, by the inhibition of transcription factor binding, as well as indirectly, by the recruitment of methyl-binding domain proteins such as MeCp2. MeCp2 binding is followed by the recruitment of a repressor protein complex which includes histone deacetylases (HDACs), and eventually leads to a closed chromatin configuration and gene silencing [10].

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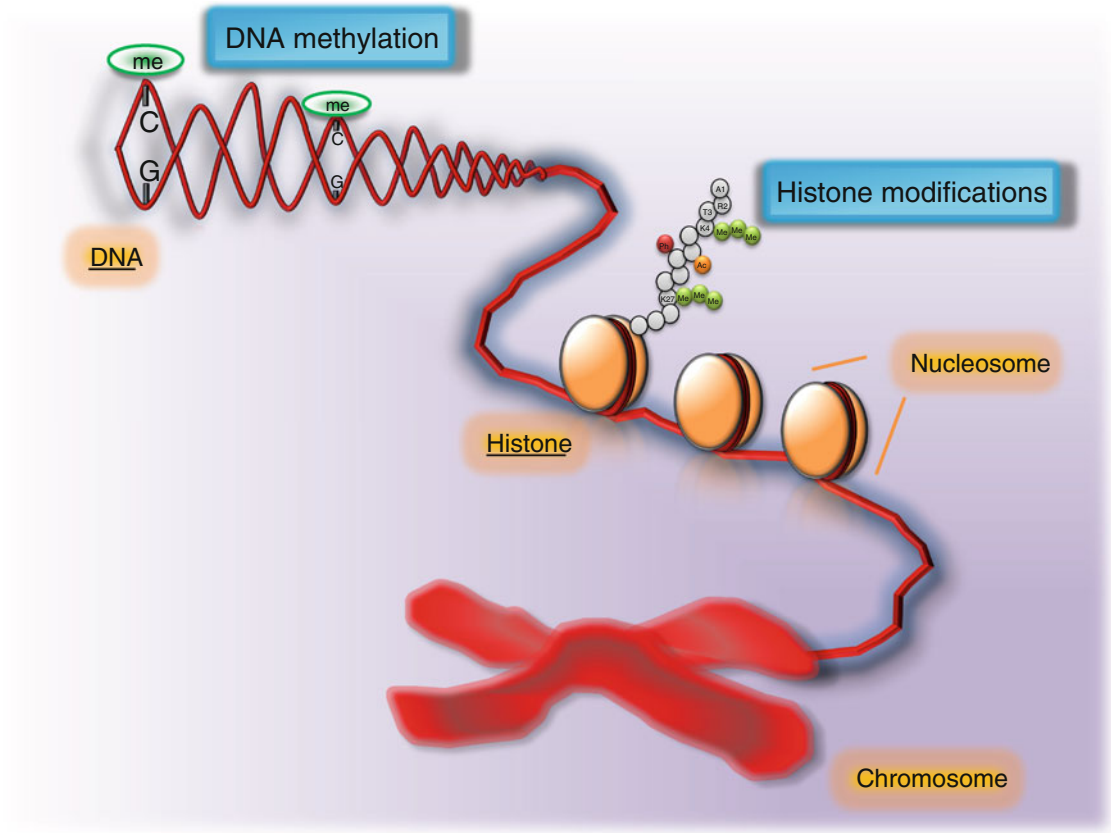

Fig. 1 DNA methylation and histone modification. DNA methylation (*green oval*) occurs at the cytosines of a CpG dinucleotide, resulting in the formation of 5-methylcytosine (5mC). Histones are small proteins which DNA wrap around to form nucleosomes. Specific modifications (denoted by *small green*, *orange*, and *red circles*) to the amino acids (denoted by *gray circles*) of these histones' tails occur

Recently, the TET protein family members TET1, TET2, and TET3 have been found to convert 5mC into 5-hydroxymethylcytosine (5hmC) [11, 12]. Further oxidation to 5hmC could also occur, resulting in the generation of the oxidation derivatives 5-formylcytosine and 5-carboxylcytosine [13]. Although the biological significance of these derivatives remains to be fully understood, they likely play an essential role in active demethylation. TET proteins show tissue-specific differential expression, with TET1 being mainly expressed in embryonic stem cells, whereas TET2 and TET3 are more ubiquitously expressed [14]. Interestingly, *TET2* has been found to be mutated in myeloid malignancies including MDS (discussed later).

Histones are small proteins which wrap around DNA to form nucleosomes. Each nucleosome consists of eight histone molecules (two each of H2A, H2B, H3, and H4) [15]. Among these histone molecules, H3 and H4 have been well studied and are considered to be key regulators of chromatin configuration, adding post-translational modifications including methylation, acetylation, phosphorylation, ubiquitination, and sumoylation to their N-terminal tails which protrude outside the basic nucleosomal structure [16–18]. Specific modifications to the amino acids in these histone tails occur, resulting in changes to the chromatin configuration. These changes serve either to promote or silence transcription, depending upon the specific amino acid(s) affected. Of these modifications, methylation and acetylation of specific lysine residues on H3 and H4 are the most studied [19]. Several enzymes have been identified which catalyze the modifications: histone acetyltransferases (HATs), HDACs, histone methyltransferases (HMTs), and histone demethylases (HDMTs). These enzymes work in concert with transcriptional activator/repressor complexes to target specific gene promoters.

Epigenetic changes in cancer

There are two aspects to changes in DNA methylation in cancer. For the most part, these changes involve simultaneous global demethylation and de novo gain of methylation at unmethylated CGIs [20]. Global DNA hypomethylation was first inferred from the measurement of global 5mC content and is now considered to be a common feature in cancer [21]. In various cancers, loss of

5mC content was found to reach an average of 10 % [22], and affected repetitive elements and specific gene promoters [23, 24]. Although the cause of this demethylation remains unclear, marked loss of 5mC was shown to be associated with chromosomal breaks, genomic instabilities, increased mutation rates, and reactivation of normally silenced genes [23, 25]. Alongside global DNA hypomethylation, many genes have been shown to demonstrate de novo DNA methylation, especially at their promoters [2]. As mentioned above, the fact that these events are linked to the silencing of gene expression leads to the hypothesis that de novo DNA methylation is an alternate way of silencing tumor suppressor genes. Evidence has been shown for CGI methylation and its tumorigenic ability in genes such as *RB1*, *p16*, *VHL*, and *MLH1* [26], suggesting a selective advantage by apoptotic deficiency and unlimited proliferation, which tumor cells can obtain as a consequence of these methylation events.

Little is known about the patterns of histone modification disruption in human tumors. Recent studies have shown a global loss of H4 lysine 16 monoacetylation and H4 lysine 20 trimethylation in cancer [27]. These modifications were found to occur throughout the genome, specifically overlapping with areas of DNA hypomethylation in repetitive sequences. Conversely, loss of H3 lysine 9 acetylation and lysine 4 dimethylation or trimethylation and gain of H3 lysine 9 dimethylation or trimethylation and lysine 27 trimethylation were found at specific gene promoters, and can contribute to tumorigenesis by silencing critical tumor suppressor genes [28].

A recent interesting observation is the correlation between DNA methylation and histone modification. A certain group of genes has been found to be marked by the repressive polycomb group (PcG) of proteins, which are complexes responsible for H3K27 trimethylation in embryonic stem cells, and further marked by DNA methylation in cancer [29, 30]. These two groups appear to overlap, implying that certain genes are "poised" for silencing and "predetermined" to be the target of specific repressive histone marks in cancer.

It has also become apparent that histone modifications are misregulated due to genes genetically altered in cancer [31]. Direct evidence of this misregulation comes from the fact that several histone-modifying enzymes are molecularly altered in cancer. For example, *MLL*—an H3 lysine 4 methylase—is rearranged in a significant portion of acute leukemias [32], and *EZH2*—an H3 lysine 27 methylase—is overexpressed or mutated in various malignancies [33, 34]. Interestingly, *UTX*, which catalyzes H3 lysine 27 demethylation, has also been identified as mutated in several types of cancers [35]. This suggests that shifting the balance of histone modifications is one of the important features of the alteration of gene expression in cancer by dysregulated histone modifiers. Dysregulations in gene expression could also occur via the recruitment of histone-modifying enzymes affected by altered genes in cancer. For example, the chimeric oncoprotein PML-PARα in acute promyelocytic leukemia is shown to target specific promoters through the aberrant recruitment of HDACs and HMTs, which leads to silencing of gene expression [36]. Recently, recurrent somatic mutations were found in genes encoding histone proteins in pediatric glioblastomas [37, 38]. These mutations result in amino acid substitutions at two critical residues in the tail of H3: lysine 27 and glycine 34, which likely results in a significant impact on chromatin structure.

Epigenetic changes in MDS

The fact that the phenotypic diversity of MDS cannot be fully explained by the recurrent aberrant karyotypes, which are well studied and used for their classification, has emphasized the importance of searching for other mechanisms responsible for this disease. Although detailed studies of histone modifications in MDS remain to be described, studies of DNA methylation in MDS have been intensively performed. A series of single locus studies have demonstrated that several genes are silenced in association with the methylation of their promoter in MDS. These include genes involved in cell-cycle regulation, apoptosis, adhesion and motility, and other pathways [26]. Among these, *CDKN2B (p15)* methylation is frequently reported in therapy-related chronic myelomonocytic leukemia, refractory anemia with excess blasts in transformation, or AML arising from MDS [39, 40]. *CDKN2B* methylation in MDS is also associated with old age, deletions of 5q and 7q, and a poor prognosis [41]. Roughly 50 % of MDS patients show silenced gene expression due to hypermethylation of *CDKN2B* at its promoter [42]. Interestingly, hypermethylation was found in rates which started at 0 % in low-risk MDS, increased to 30 % in high-risk MDS, and reached 75 % in AML transformed from MDS [40]. In a recent study focusing on quantitative analysis of the DNA methylation status of ten selected genes [43], a hypermethylator phenotype of CGIs was identified that marks a subset of cases with MDS which often show concordant hypermethylation of several genes. This phenomenon, called CpG island methylator phenotype (CIMP), was first described in colon cancer [44] and later in glioma [45]. It results in the simultaneous inactivation of several genes by an unknown mechanism. In MDS, CIMP is associated with rapid progression to AML and shortened overall survival and progression-free survival [43]. This explains in part why the methylation of so many genes is reported as prognostic in MDS [26]: all these studies of individual genes are likely a common subset of cases affected by CIMP. Importantly,

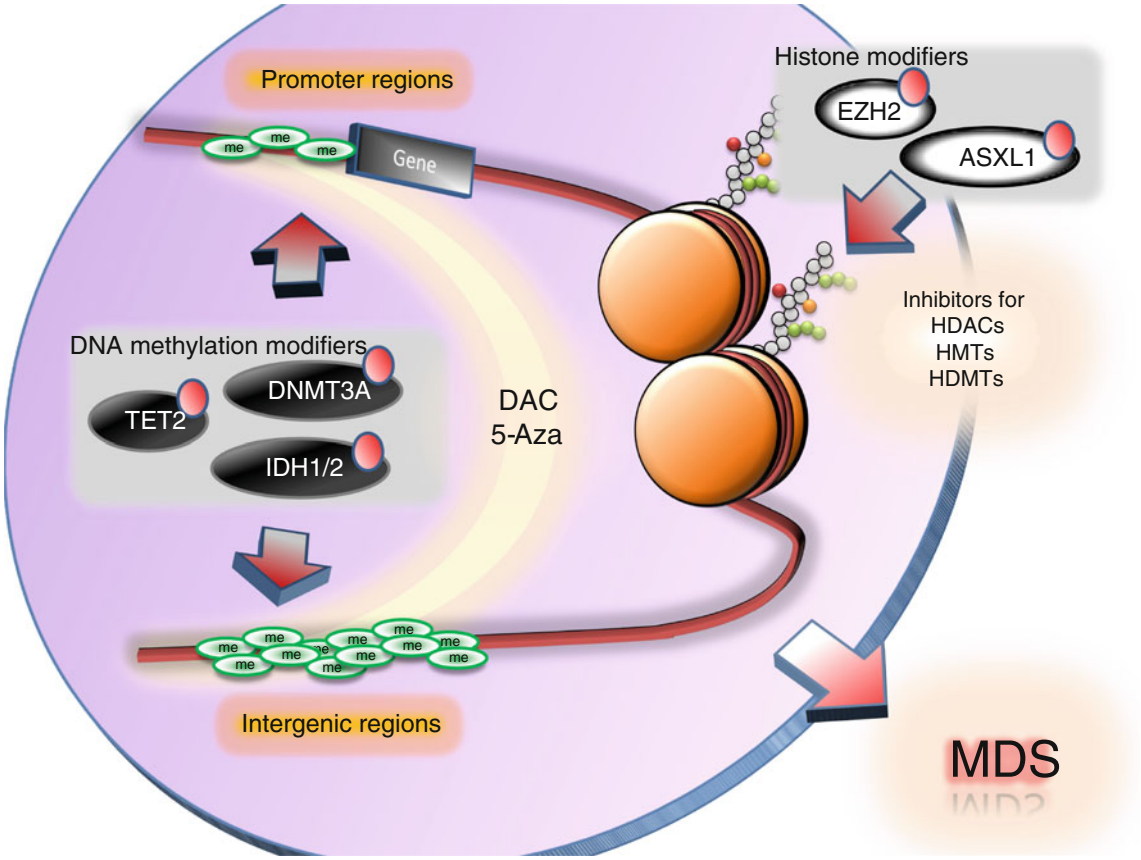

Fig. 2 Mutations of epigenetic regulators and possible targeted therapies. Various mutations have been identified in the genes of epigenetic regulators. These mutations are assumed to confer growth advantages to cells, resulting in MDS by affecting DNA methylation (in promoter and intergenic regions) and histone modification. DAC and AZA inhibit these effects on DNA methylation, whereas inhibitors for HDACs, HMTs, and HDMTs work on histone modification for the treatment of MDS

some of these genes clearly have minimal functional impact on MDS, because they are not expressed in normal hematopoietic cells.

Genome-wide studies of DNA methylation have revealed that hundreds of genes are frequently hypermethylated in MDS, including genes of the WNT signaling pathway and MAP kinase pathway [46]. It has also been suggested that aberrant DNA methylation occurs more frequently than chromosome lesions in MDS [47]. As indicated by studies of individual genes, hypermethylation across the genome is associated with poor prognostic features and transformation to AML, independent of chromosomal aberration. There was a distinct methylation pattern in MDS and related AMLs in comparison to de novo AML, pointing to distinct pathogenic mechanisms [46]. As a result, DNA methylation is considered to be abnormal early on in MDS, and progression of the disease is associated with the accumulation of additional epigenetic events. These observations, taken together with the fact that DNA methylation is a reversible process, provide a rationale for the use of DNA methylation inhibitors in the treatment of MDS (discussed later).

Compared to hypermethylation, global hypomethylation in MDS is less well understood. LINE-1 was used as a surrogate marker for global DNA methylation, and it was found to be increased in MDS [48] as opposed to solid tumors where decreases are often seen [23]. Using a promoter-array for methylation analysis, a smaller number of hypomethylation sites was observed compared to hypermethylation sites in MDS [46]. A recent report showed frequent promoter hypomethylation in *TET2* mutant CMML cases [49], though this was not consistently found in other studies [50]. Extensive genome-wide analysis will be required to study global hypomethylation in MDS occurs to the same extent as in the other cancers.

Recent advances in technologies such as high-resolution SNP array and next-generation sequencing have led to important new findings in MDS. Various mutations have been identified in the genes that code for epigenetic regulators, including *ASXL1*, *DNMT3A*, *EZH2*, *IDH1/2*, and *TET2* [51] (Fig. 2; Table 1). Although it is not yet fully understood whether MDS cases with mutations in these genes have characteristic epigenetic patterns, these findings suggest that epigenetic dysregulation has strong

Table 1 Mutations in epigenetic regulators in MDS

Gene	Frequency	Function	References
TET2	20 % in MDS	Conversion of 5mC to 5hmC	[52, 82]
	30–50 % in CMML		
IDH1/2	<10 % in MDS	Conversion of isocitrate to αKG	[83]
		Mutants generate 2HG from αKG	
DNMT3A	<10 % in MDS	De novo DNA methyltransferase	[84]
ASXL1	10–20 % in MDS	Interaction with histone modifiers	[85, 86]
	>40 % in CMML		
EZH2	<10 % in MDS	Histone methyltransferase (H3K27me3)	[86]

implications in this disease. *TET2* is one well-studied gene in this regard. The incidence of *TET2* gene alterations ranges from 10 to 25 % in myeloid malignancies, with the highest frequency of mutation found in CMML, where *TET2* mutations are noted in 35–50 % of cases [52–54]. As reported for TET1 [12], TET2 also converts 5mC to 5hmC [11] in embryonic stem cells, and thus mutations of *TET2* were theorized to contribute to leukemogenesis and to the disruption of hematopoietic differentiation by altering the epigenetic regulation of transcription via DNA methylation [49, 55]. Furthermore, in murine models, *TET2* deficiency impairs hematopoietic differentiation with the expansion of myeloid precursors [56, 57]. The exact mechanism and the extent to which *TET2* mutations affect DNA methylation remain in question. Ko et al. [49] reported a subset of genes with hypomethylation in CMML patients with *TET2* mutations. However, our data recently suggested that effects of *TET2* mutations on DNA methylation are primarily outside both CGI and promoters ([50] and in submission). These findings may provide further understanding of CMML leukemogenesis and could lead to the development of new strategies for CMML patients.

Epigenetic therapy

Epigenetic therapy refers to the treatment of cancer by targeting epigenetic pathways [58]. The principal idea of this approach is to pharmacologically relieve the effects of DNA methylation and chromatin remodeling on silenced genes in malignant cells. There are two classes of drugs that modify epigenetics which have been approved by the US Food and Drug Administration (FDA) for the treatment of cancer: DNA methylation inhibitors and HDAC inhibitors (HDIs).

Two cytosine analogs were first developed as cytotoxic agents in 1960s, found to induce peculiar differentiation phenotypes in vivo in 1970s, and shown in the early 1980s to be potent DNA methylation inhibitors [59]. The DNA hypomethylating property is limited to cytosine analogs with the shared structure of 5′ modifications of the ring. This property has been studied in two main analogs, 5-azacytidine (azacitidine, or AZA) and 5-aza-2′-deoxycytidine (decitabine, or DAC), because of their ability to incorporate into DNA and trap DNMTs through an irreversible covalent bond, leading to degradation of these enzymes [60]. Hypomethylation occurs in the cells proliferating after DNA synthesis in the absence of these enzymes, leading to hypomethylation in the daughter cells.

AZA incorporates into RNA, or incorporates into DNA after intracellular conversion to DAC and subsequently inhibits DNA methylation. Unlike AZA, DAC does not incorporate into RNA and is directly incorporated into DNA [61]. These drugs were found to be more effective in hematologic malignancies than in solid tumors, but are also quite toxic [62]. Interestingly, they only work as epigenetic modifiers when given at low doses. High doses inhibit DNA synthesis, which precedes their DNA hypomethylating effect. Recent work suggests that low doses of these agents hold the key to therapeutic benefits even in epithelial tumors [63]. One might easily imagine that initial studies of these drugs failed because of their unusual dose–response properties. However, AZA and DAC re-emerged after evidence of their effectiveness in the treatment of older patients at low dose was found [64, 65]. Both AZA and DAC were tested later in relatively large studies at low to moderate doses and over multiple cycles of administration, thus optimizing their epigenetic modulation potential. Following promising phase II studies, AZA was tested in two separate phase III studies [66, 67] in MDS. Response rates ranging from 30 to 60 % were observed, with improved survival as compared to either supportive care or cytotoxic chemotherapy. DAC also had promising early studies in MDS, and phase II studies confirmed responses (40 % complete response; over 70 % total response) and substantial effects on survival [68, 69]. While there is relatively little data regarding their use in low-risk MDS, these two agents have been a major breakthrough and have become the standard of care for high-risk MDS, though a direct comparison between the two agents is not available.

One of the peculiar characteristics of epigenetic therapies is that the patterns of response are quite different from traditional cytotoxic therapies in MDS. In contrast to chemotherapy (which induces rapid response in MDS), a remarkable response induced by AZA and DAC is rare after one cycle, but is improved over time [66, 67] and with continued therapy [70]. Hypomethylating agents show

mainly reversible side-effects on myelosuppression rather than the usual cytotoxic side-effects (mucositis, hair loss, diarrhea, renal failure, etc.). Most importantly, hypomethylating agents seemed to show better results when compared with traditional chemotherapy [67, 69], even low-dose chemotherapy. Furthermore, combinations of these drug and conventional chemotherapy have also been of clinical interest, since there are encouraging results in AML [71], and some potential in MDS [72]. Several studies are currently ongoing to obtain improved efficacies of these drugs, including the development of oral preparations [73] and small molecules that can inhibit DNMTs without requiring DNA incorporation [74].

The inhibition of histone-modifying enzymes will be another potential epigenetic target for MDS therapy. There are HDIs currently in clinical trials; however, less data is available for their use in MDS, though anecdotal responses have been reported [75]. Recently, it has also been reported that DNA methylation per se is not a permanent lock for silencing gene expression, but rather a combination of DNA methylation inhibitors with other drugs (such as HDIs) can be used to reactivate gene expression [76, 77]. Furthermore, there is increasing evidence that such combinations have encouraging results [78, 79]. Therefore, it is likely that the rational design of combinations of several drugs targeting epigenetic pathways will be required for the treatment of MDS (Fig. 2). There is also a particular interest in developing drugs that can inhibit the activity of other epigenetic pathways, such as HMTs [80, 81], because these could work independently of (and could complement) DNA methylation inhibitors and HDIs. Given that mutations in several epigenetic regulators have been identified, it will be interesting to see if patients with different statuses for these mutations show different responses to epigenetic therapies.

Conclusion

There is increasing evidence that epigenetic mechanisms have been associated with gene expression variability in MDS. In addition to chromosomal aberrations, epigenetic aberrations have been found to play an important role in establishing the heterogeneous phenotype of MDS. Recent findings from genome-wide studies have revealed several mutations in epigenetic regulator genes and peculiar patterns in epigenetic status, emphasizing the importance of understanding the underlying epigenetic mechanisms of this disease. Further investigation will help provide new insight into the classification, prognosis, and treatment of MDS.

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