HBV cccDNA的表观遗传学修饰及调控

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摘要 乙型肝炎病毒(hepatitis B virus, HBV)感染是一个重大的世界性公共卫生问题. HBV能够引起急性或慢性病毒性肝炎, 最终导致肝硬化甚至肝癌的发生. 现行的临床抗HBV药物, 如 α 干扰素和核苷(酸)类似物等, 虽可有效抑制病毒复制, 但不能彻底清除病毒. 其根本原因在于, HBV感染肝细胞后形成共价闭合环状DNA(covalently closed circular DNA, cccDNA), 并以微染色体的形式独立稳定存在于肝细胞核内. 表观遗传学修饰可在不改变DNA序列的情况下, 影响基因的表达. 越来越多的证据表明, cccDNA的表观遗传学修饰是调节HBV生活周期的重要因素. 本文就HBV cccDNA的表观遗传学修饰和调控作用、表观遗传修饰药物和治疗, 以及表观遗传学研究方法和体系等进行综述.

关键词 乙型肝炎病毒, 共价闭合环状DNA, 表观遗传学修饰, 甲基化, 乙酰化, 表观遗传学治疗

乙型肝炎病毒(hepatitis B virus, HBV)感染至今仍然是一个重大的世界性公共卫生问题. HBV感染人体后, 可造成急性或慢性病毒性肝炎. 急性病毒性肝炎在及时接受治疗和护理后大都可以在较短时间内痊愈, 对人体没有长期危害. 而慢性病毒性肝炎(chronic hepatitis B, CHB)不仅病程长、极难治愈, 且在漫长的治疗过程中有向肝纤维化、肝硬化甚至肝癌(HCC)转化的风险^[1]. HBV是一种有囊膜的DNA病毒, 在其生活周期中, HBV DNA于肝细胞核内形成共价闭合环状的DNA(covalently closed-circular DNA, cccDNA), 进而与宿主细胞的组蛋白(histones)、非组蛋白(nonhistone proteins)以及病毒核心蛋白(HBc)、X蛋白(HBx)等结合, 以类似于细胞基因组染色质样结构的微染色体形式独立存在于细胞核内^[2~5]. 微染色体结构稳定, 可长期存在^[6], 无论是人体自身还是现有抗乙肝药物都难以将其彻底清除, 这也是CHB难以治愈的根本原因.

正因为cccDNA在慢性乙肝病程中有着特殊的地

位, 所以cccDNA的形成和调控机制成为了目前乙肝方面的研究热点. 本文综述了HBV cccDNA的表观遗传学修饰及其调控作用、表观遗传学修饰药物和治疗, 以及表观遗传学研究方法和体系等.

1 DNA甲基化

DNA甲基化在哺乳动物中通常发生在CpG岛中胞嘧啶环的5'位置, 由DNA甲基转移酶(DNA methyltransferases, DNMTs)介导, 一般与转录沉默有关^[7]. 经预测, HBV cccDNA中含有3个CpG岛. 如图1所示, CpG I横跨过HBV S基因的起始位点; CpG II包含一部分增强子I(enhancer I)、X基因启动子(XP)和一部分Core基因启动子(CP); CpG III则与HBV P基因的起始位点区域重叠^[8,9]. 在HBV已经明确的8个基因型(A~H)和2个尚未明确的基因型(I, J)中, CpG I只存在于A, B, D, E, I这5个基因型中. 而CpG II和CpG III存在于A~J等所有基因型中^[8]. 3个CpG岛中, CpG I的甲基化相对少见^[10], 对

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图1 (网络版彩色)HBV基因组结构示意图。HBV基因型: A型(GenBank: AB116086)。由内而外分别是, 预测的CpG岛: CpG I, CpG II和CpG III; 增强子: Enh I和Enh II; 启动子: SP1, SP2, CP, XP; 开放阅读框(ORF): preC/Core, 聚合酶(pol), preS/S和X

Figure 1 (Color online) Schematic illustration of the HBV genome. The sketch of the HBV genome (genotype A, GenBank: AB116086), which includes three predicted CpG islands (CpG I, CpG II and CpG III), the regulatory elements (enhancer I [Enh I] and enhancer II [Enh II]), the promoters (SP1, SP2, CP and XP) and the open reading frames (ORFs: preC/Core, polymerase [pol], preS/S and X)

cccDNA转录及病毒复制的影响尚未见相关报道。CpG II的甲基化和CHB病人的低病毒血症有关, 体外转染实验表明, CpG II的甲基化可导致HBV 3.5 kb RNA转录和病毒DNA复制水平的下降, 而HBV 2.4/2.1 kb RNA水平不受影响^[10]。CpG III的甲基化与病人血清中HBV S抗原水平降低有关^[10], 此外, 与肝炎和肝硬化的组织相比, 肝癌组织中CpG III的甲基化明显升高^[11], 提示着CpG III的甲基化可能与肝癌的发生有关。

目前, DNMTs对HBV DNA甲基化的具体调控作用还不清楚。尽管有体外感染实验表明, 被HBV感染的肝细胞中, 所有3种DNMTs(DNMT1, DNMT2, DNMT3)的表达水平均明显上升^[12], 但其中的机制尚未可知。此外, DNMTs的上调虽然可以造成HBV DNA的高甲基化, 使得HBV复制能力下降, 但是宿主基因同样会被甲基化而受到影响^[12]。因此, DNMTs并不具备cccDNA选择性。

2 组蛋白修饰

组蛋白是组成染色体的基本结构蛋白。人体内的

组蛋白分为5种: H1, H2A, H2B, H3和H4。核小体是组成染色体的基本结构单位, 由DNA缠绕核心八聚体形成。每2个H2A, H2B, H3和H4(共8个)共同组成核心八聚体, 因此这4种组蛋白也被称为核心组蛋白。核心组蛋白可以进行大量的翻译后修饰, 包括乙酰化、甲基化、磷酸化和泛素化等^[2]。与宿主细胞的染色质类似, 核心组蛋白也参与了cccDNA微染色体的组成。因此, 这些组蛋白修饰能通过不同途径在不同程度上调控HBV的转录和复制。其中, 乙酰化和甲基化是报道最多、研究最为深入的两种修饰。

2.1 乙酰化

蛋白质乙酰化是指将乙酰基转移到氨基酸侧链基团上的过程。最常见的组蛋白乙酰化由组蛋白乙酰转移酶(histone acetyltransferases, HATs)催化发生, 去乙酰化则由组蛋白去乙酰化酶(histone deacetylases, HDACs)催化发生。核心组蛋白属于碱性蛋白质, 富含精氨酸、赖氨酸等碱性氨基酸, 在生理条件下带正电, 可与带负电的DNA相互吸引、紧密结合, 导致核小体构象紧凑, 染色体异染色质化。乙酰化可减弱组蛋白与DNA之间的相互作用, 使得染色质构象变得松散, 有利于转录调节因子接近DNA, 从而促进基因转录。相应地, 去乙酰化可抑制基因转录。研究显示, 慢性乙肝病人肝组织内, cccDNA结合的组蛋白H3, H4的乙酰化程度与cccDNA的转录活性密切相关, 乙酰化程度高则转录活性高, 乙酰化程度低则转录能力下降^[13]。GCN5和PCAF是一对高同源性HAT, 主要催化H3K9的乙酰化^[14]。P300和CBP是另一对同源性极高的HAT, 主要催化H3K18和H3K27的乙酰化^[14-16]。去乙酰化酶中, HDAC1可去乙酰化H3和H4, 而SIRT3主要去乙酰化H4^[13,17]。

HBx蛋白作为一个HBV病毒蛋白, 对HBV的转录和复制有重要的调节作用。体外转染实验显示, HBx的存在招募了一系列乙酰转移酶(p300/CBP, GCN5/PCAF等)到cccDNA上, 从而提高了组蛋白的乙酰化水平, 进而促进HBV转录和复制^[15]。而在HBx缺失的时候, cccDNA上的乙酰转移酶数量明显减少, 而去乙酰化酶(HDAC1, SIRT1等)变多, 相应导致组蛋白乙酰化水平降低, 转录水平下降^[5,17]。此外, HBV感染人肝嵌合小鼠实验显示, α 干扰素处理的小鼠体内cccDNA结合的组蛋白H4的乙酰化明显减少^[18], 这暗示着 α 干扰素作为现有的治疗慢性乙肝的重要手段, 同样可以通过调节

cccDNA组蛋白乙酰化水平来达到控制HBV的目的.

2.2 甲基化

与组蛋白乙酰化主要正向调控转录活性不同, 组蛋白的甲基化既可能导致转录活化, 也可能导致转录抑制. 组蛋白中的赖氨酸(lysine, K)可结合1, 2或3个甲基基团(methyl group), 形成单甲基化、双甲基化或三甲基化修饰. 精氨酸(arginine, R)可发生单甲基化、对称双甲基化和非对称双甲基化等3种修饰^[19]. 研究发现, 组蛋白H3的4, 36, 79位赖氨酸的三甲基化(分别是H3K4me3, H3K36me3, H3K79me3)与转录活化相关, 而9, 27位赖氨酸的甲基化(分别是H3K9me2, H3K9me3, H3K27me3)与转录抑制相关^[20~23]; 而组蛋白H4第3位精氨酸的非对称双甲基化(H4R3me2a)与转录活化有关, 对称双甲基化(H4R3me2s)则与转录抑制有关^[24].

组蛋白甲基化由组蛋白甲基转移酶(histone methyltransferases)和组蛋白去甲基化酶(histone demethylases)调控, 它们中有一些已被报道参与了cccDNA的表观遗传学调控. 例如, PRMT1(arginine methyltransferase 1)是HBV的负调节因子, 可以被招募到cccDNA上, 同时和HBx有相互作用^[25]. HBx可通过与PRMT1的相互作用减弱PRMT1造成的蛋白质甲基化, 从而解除PRMT1对HBV的抑制效果^[25]. PRMT5通过催化cccDNA结合的H4R3me2s来抑制HBV转录^[26]. Spindlin1可被招募到cccDNA上, 降低cccDNA结合的H3K4me3从而抑制HBV转录^[27]. 类似的还有SETDB1^[28], SETD1A, EZH2, SUV39H1^[17]等, 这些调节因子的调控作用均在HBV体外感染系统中得到了验证.

3 病毒蛋白对cccDNA转录活性的调控

3.1 HBc

多项研究显示, HBc可以被招募到cccDNA上^[4,6,29], 并且更倾向于结合CpG II, 减少CpG II的甲基化^[29]. 有趣的是, 和感染了野生型病毒的鸭肝细胞相比, 感染了HBc缺陷的鸭乙型肝炎病毒(DHBV)的细胞中, 病毒mRNA水平明显下降, 这似乎暗示着HBc参与调节cccDNA的转录活性^[30]. 但是在人肝癌细胞HepG2中, 无论感染的是野生型还是HBc缺陷型病毒, HBV mRNA水平都是相似的^[10]. 因此, HBc是否对cccDNA有调节作用还有待进一步研究.

待进一步研究.

3.2 HBx

HBx是HBV的调节因子, 对起始和维持病毒复制尤为重要^[31]. 如图2所示, HBx的存在可促进HAT与cccDNA的结合, 同时减少cccDNA上的HDAC, 使得cccDNA结合的组蛋白乙酰化增加, cccDNA转录活性上升^[5]. 在HBx缺失的情况下, 转录抑制因子如SETDB1, PRMT1, Spindlin1等结合到cccDNA上, 导致cccDNA结合的H3K9me2/3增加, H3K4me3下降, 转录活性下降^[25,27,28]. HBx的缺失还会使得异染色质蛋白HP1在cccDNA上的募集增加, 导致染色质结构变得更加紧密, 阻碍转录进行^[28]. 此外, 体外感染实验显示, Smc5/6作为一个染色体结构维持复合物可以抑制cccDNA的转录, 而HBx可以劫持DNA损伤结合蛋白1(DNA damage-binding protein 1, DDB1), 并招募CRL4(cullin 4A-RING E3 ubiquitin ligase)形成CRL4-DDB1复合物, 进而泛素化Smc5/6使其降解, 从而促进cccDNA的转录活性^[32~34].

4 表观遗传学修饰药物

表观遗传学修饰在许多癌症或非癌症疾病的发生和维持中扮演着十分重要的角色^[35], 因而受到科学家、临床医生和医药行业的广泛关注. 目前, 肿瘤学中最前沿的表观遗传学治疗方法包括DNMT抑制剂(DNMTi)和HDAC抑制剂(HDACi). 阿扎胞苷(azacitidine)和地西他滨(decitabine)是经美国FDA(Food and Drug Administration)认证的两种DNMTi类药物, 主要用于治疗骨髓增生异常综合征(MDS)、非淋巴细胞白血病等血液疾病^[36,37]. 值得注意的是, 阿扎胞苷有潜在的肝毒性, 不适用于有肝损伤的病人^[38]. 相比之下, HDACi类药物的开发更为普遍, 上市药物也更多, 例如伏立诺他(vorinostat)、罗米地辛(Romidepsin)、贝利司他(Belinostat)、帕比司他(Panobinostat)和西达本胺等^[39]. 和DNMTi一样, HDACi主要治疗T细胞淋巴瘤和多发性骨髓瘤等血液病, 对实体瘤没有明显疗效^[40,41]. 此外, 这两类药物均属于广谱抑制剂, 即对大部分DNMTs和HDACs都有抑制效果, 缺乏基因特异性, 因而具有较大的毒副作用. 目前有关表观遗传学治疗的研究热点除了开发具有特异性、选择性的表观遗传学新药外, 还包括将表观遗传学疗法与化学疗法、免疫疗法等联合使用, 达到增敏减毒的效果.

图 2 (网络版彩色)HBV cccDNA相关组蛋白修饰示意图。(a) HBx存在时,多种转录促进因子(如CBP/p300, GCN5/PCAF)与cccDNA结合,cccDNA微染色体结构变得松散,转录活性增高;(b) HBx缺失时,cccDNA结合的转录促进因子减少,而转录抑制因子(如HDAC1, PRMT1, SETDB1)增加,微染色体异染色质化,结构变得紧密,cccDNA转录活性降低

Figure 2 (Color online) Schematic illustration of the HBV cccDNA-bound histones modification. (a) In the presence of HBx, transcriptional activation factors, such as p300/CBP and GCN5/PCAF, are recruited to cccDNA and hyperacetylate the cccDNA-bound histones. Viral minichromosome is in a relaxed configuration and has higher transcriptional activity. (b) In the absence of HBx, cccDNA-bound HATs are severely impaired, while HDACs and other transcriptional restriction factors are recruited to cccDNA, leading to a tight structure of minichromosome with lower transcriptional activity

目前已施行的或处于临床试验阶段的抗HBV药物包括干扰素(IFN)、逆转录酶抑制剂(核苷类似物)、蛋白衣壳变构调节剂、病毒侵入或释放抑制剂等,但这些药物对细胞内稳定存在的cccDNA的作用十分有限^[42,43]。表观遗传学领域的研究为HBV的抗病毒治疗开辟了新的方向。如上所述, DNMTs和HDACs有抑制HBV转录活性的作用,且HDACi已在体外转染实验中被证明可以促进HBV的转录和复制^[13]。因此,现有的DNMTi和HDACi类药物可能并不适用于治疗HBV。目前已有部分去乙酰化酶和甲基转移酶(如Sirt3和PRMT5)在体外感染实验中被证明可改变cccDNA结合的组蛋白的相关修饰,而不影响宿主基因的表达^[17,26]。因此,研发这类特异性影响HBV的HDACs或PRMTs的活化剂,以及联合使用免疫调节剂(如IFN)等,有望在HBV相关肝脏疾病的治疗中取得更好的效果。随着有关研究的深入和新药的研制,HBV表观遗传学治疗有着乐观的前景。

5 cccDNA表观遗传学研究方法与体系

染色质免疫共沉淀(chromatin immunoprecipita-

tion, ChIP)是目前研究表观遗传学的主要方法。其基本原理为:用甲醛固定活细胞内的蛋白质-DNA复合物,通过超声或酶切法将其切断为一定长度的染色质片段,加入特异性抗体进行免疫沉淀,富集目的蛋白。而后对目的蛋白上结合的DNA片段进行纯化和检测,从而获得蛋白质与DNA相互作用以及蛋白质修饰和基因表达的关系等信息^[13]。除了可以独立使用外,ChIP技术还能与其他方法结合,扩大应用范围。例如将ChIP和第二代测序结合而成的ChIP-seq技术,以及将ChIP和基因芯片结合的ChIP-chip技术,都是适合高通量筛选的常用方法。

自然感染状态下,每个肝细胞内的cccDNA量很少,而这也成为应用ChIP技术研究HBV cccDNA的主要障碍。在现有的HBV研究体系中,来源于人肝组织的原代肝细胞(primary human hepatocytes, PHH)或HBV易感细胞系(如HepaRG)是研究HBV感染最理想的细胞模型。但这些材料来源稀少、成本昂贵,因而限制了它们的应用。近年来,得益于HBV功能性受体牛磺胆酸钠共转移多肽(NTCP)的发现^[44], HepG2-NTCP, Huh7-NTCP等更加廉价易得的HBV体外感染细胞模型日趋成熟。

此外, HepAD38细胞基因组中嵌入了HBV基因组, 能支持完整的HBV生活周期, 并形成稳定的cccDNA池^[45]. 以上HBV研究系统各有优劣, 给研究人员提供了更多选择性, 但它们都没能解决cccDNA含量低的问题. 在实际应用中, 往往需要通过增加细胞总量等方法来提高cccDNA的获得量, 试剂耗材等研究成本随之增加, 实验结果的高背景也难以避免. 如果是研究组蛋白等与cccDNA结合的高丰度蛋白, 这些系统尚可满足条件; 但要用于研究低丰度蛋白或与cccDNA瞬时结合的蛋白, 如转录因子、信号通路分子等就比较困难了. 因此, 人工模拟cccDNA系统亟待开发.

2006年, Pollicino等人^[13]建立了线性单倍体HBV转染细胞后在细胞内进行自环化的系统, 并将其运用于cccDNA的表观遗传学研究. 研究人员首先构建了含有单倍体HBV基因组的克隆载体, 再通过单酶切得到首尾末端互补的线性单倍体HBV DNA, 随后转染细胞. 线性单倍体HBV DNA在细胞内发生自环化, 形成cccDNA. 实验结果表明, 该系统内cccDNA含量在转染后12~48 h处于高峰期, 约为自然感染状态下cccDNA含量的10倍. 迄今为止, 研究人员已经利用该系统验证了HBx, HDAC1, CBP, IFN等多种因子对HBV cccDNA的表观遗传学调控作用^[5,13,18]. 2014年, 通过引入Cre-LoxP

重组酶系统, Qi等人^[46]建立了一种重组cccDNA研究系统. 研究人员构建单倍体HBV克隆载体(prcccDNA)时, 在HBV基因组两端各加一个LoxP位点, 随后将该质粒和表达Cre重组酶的pCMV-Cre质粒共转染. Cre重组酶在细胞内识别prcccDNA上的LoxP位点后将其切割重组, 形成比野生型cccDNA多约140 bp内含子的重组cccDNA(rcccDNA). 研究结果显示, rcccDNA有着与野生型cccDNA类似的微染色体结构和表观遗传学特征, 并能支持完整的HBV复制周期. 此外, 当该系统中两种质粒比例恰当时, prcccDNA可被完全切割为rcccDNA, 极大地提升了cccDNA的获得量. 这些人工系统的开发为cccDNA表观遗传学研究提供了有力支持.

6 总结

cccDNA在HBV生活周期中的地位决定了它是攻克慢性乙型肝炎的关键. 干扰素和核苷类似物是目前仅有的两种已上市的治疗HBV的有效药物, 但他们对cccDNA的清除作用都有限. 有关cccDNA表观遗传学修饰的研究为发展抗乙肝药物提供了一个新的思路, 尽管现有的表观遗传学药物似乎并不适用于治疗HBV, 但新的具有特异性的表观遗传学药物的研发以及审慎的临床观察可能给治愈HBV带来新的希望.

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Summary for “HBV cccDNA 的表观遗传学修饰及调控”

Epigenetic modification and regulation of HBV cccDNA

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Hepatitis B virus (HBV) infection, which can cause acute or chronic viral hepatitis, hepatic fibrosis, cirrhosis, and carcinoma (HCC), is a major global health problem. After the virus enters a hepatocyte, its genome translocates into the cell nucleus and forms covalently closed circular DNA (cccDNA). HBV cccDNA binds with histones and nonhistone proteins, forming an episomal minichromosome, which underlies the molecular basis of HBV infection persistence and reactivation after antiviral treatment.

The term “epigenetics” traditionally refers to heritable traits without changes in DNA sequence, but it also describes general studies of chromatin biology. Increasing evidence suggests that the epigenetic regulation of cccDNA is closely related to viral replication and the outcome of chronic HBV infection. In this review, we mainly discuss the following two mechanisms underlying epigenetic regulation: DNA methylation and histone modification.

DNA methylation usually occurs in the CpG dinucleotides; it is mediated by DNA methyltransferases (DNMTs) and is generally associated with transcriptional silencing. There are three predicted CpG islands in the HBV genome. CpG island 1 is rarely methylated, the methylation of CpG island 2 leads to reduced viral 3.5 kb RNA transcription and DNA replication, and the methylation of CpG island 3 is significantly correlated with hepatocarcinogenesis. The exact roles that DNMTs play in HBV DNA methylation have not been completely elucidated.

The most studied histone modifications are histone acetylation and methylation. Histone acetylation, which is generally associated with transcriptional activation, is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone methylation, which is associated with both transcriptional activation and repression, is controlled by histone methyltransferases (HMTs) and histone demethylases (HDMs). Some of these enzymes, such as p300/CBP, HDAC1 and PRMT5, have been reported to participate in the epigenetic regulation of HBV cccDNA.

Viral proteins are also involved in the epigenetic regulation of HBV cccDNA. HBV core protein (HBc) is a structural protein in the HBV capsid. It has been reported that HBc can be recruited to cccDNA, preferentially binding to CpG island 2. In addition, relative abundances of HBc binding are negatively associated with DNA methylation levels in CpG island 2, which suggests that HBc may be an epigenetic regulatory factor of HBV. HBx is a significant HBV regulatory protein and can also be recruited to cccDNA. In the presence of HBx, transcriptional activation factors such as p300/CBP and GCN5/PCAF are recruited to cccDNA and hyperacetylate the cccDNA-bound histones. This results in an open configuration of the viral minichromosome with higher transcriptional activity. In the absence of HBx, cccDNA-bound HATs are severely impaired, while HDACs and other transcriptional restriction factors are recruited to cccDNA, leading to a tight structure of the minichromosome with lower transcriptional activity.

In this review, we summarize the development of epigenetic therapies involving existing and emerging epigenetic drugs and their potential applications in chronic HBV infection and HBV-related HCC. In addition, we describe current techniques and systems for research on cccDNA epigenetic regulation. The discovery that epigenetic regulators play important roles in the persistence of HBV and are potential therapeutic targets has presented a myriad of opportunities to treat or even cure HBV.

hepatitis B virus, covalently closed circular DNA, epigenetic modification, methylation, acetylation, epigenetic therapy

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