HBV cccDNA检测方法的研究进展

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摘要 目前全球约有2.57亿慢性乙型肝炎病毒(hepatitis B virus, HBV)感染者, 是当前最为突出的公共卫生问题之一。HBV感染的肝细胞内稳定存在的共价闭环环状DNA(cccDNA)是造成HBV感染持续、抗病毒治疗停药后病毒反弹的重要原因。现有Anti-HBV药物难以有效清除或直接抑制肝内cccDNA是慢性乙肝难以治愈的关键瓶颈。缺乏简单易行的cccDNA定量检测方法, 影响了靶向cccDNA的Anti-HBV新药研发进展, 也阻碍了这一指标在慢性乙肝患者临床管理实践中的应用。Southern blot被认为是目前cccDNA实验室检测的“金标准”, 但其操作复杂且灵敏度低, 不适用于高通量药物筛选及临床应用。基于PCR的cccDNA检测方法相对操作简单、检测灵敏度更高, 但其可靠性仍有待考验。本文系统整理分析了cccDNA样品提取和测定方法的研究现状与进展, 希望通过综合比较不同cccDNA检测技术的性能和特点, 为cccDNA相关基础研究、药物发展与临床研究提供方法学参考。

关键词 乙型肝炎病毒, 共价闭环环状DNA, 抗乙肝药物, cccDNA检测方法

乙型病毒性肝炎, 简称乙肝, 是一种由乙型肝炎病毒(hepatitis B virus, HBV)感染机体后所引起的疾病。据世界卫生组织估计, 全世界人口中有3.5%(2.57亿)感染了乙型肝炎病毒, 每年新增感染约500万人。在如此庞大的感染人群中, 据估计, 全球每年有超过80万人死于包括慢性活动性肝炎(chronic active hepatitis)、乙肝相关肝硬化(liver cirrhosis)及原发性肝癌(hepatocellular carcinoma)等在内的各类由乙肝病毒感染引起的肝脏疾病^[1]。我国是乙肝病毒感染的中高流行地区, 尽管乙肝疫苗的广泛接种已经有效减少了新发HBV感染数量, 但据估计全国仍有7000余万慢性HBV感染者, 其中慢性乙型肝炎(chronic hepatitis B, CHB)患者达2000~3000万, 每年约50万人死于HBV感染, 每年新发乙肝病例约50~100万例^[2,3]。乙肝病毒感染严重危害了人类的健康, 带来了长期的疾病负担, 是我国现阶段最为突出的公共卫生问题之一。目前临床常用的CHB治

疗一线药物主要包括长效干扰素(PEG-IFN)和恩替卡韦、替诺福韦两种核苷/核苷酸类似物(NAs), 这些药物已被证明能抑制病毒复制(NAs类抑制HBV复制作用相比PEG-IFN更强; PEG-IFN诱导HBeAg/HBsAg血清学转换的效果要优于NAs类), 延缓/阻止疾病进展, 降低LC/HCC的发生风险^[4-6]。然而, 当前临床使用的PEG-IFN/NAs药物及其优化组合仍难以实现高比例的“临床治愈”(使得患者血清学HBsAg检测不出, <0.05 IU/mL; 伴随或不伴随乙肝表面抗体阳转)。现有研究认为, CHB患者被HBV感染的肝细胞内稳定存在的共价闭环环状DNA(covalently closed circular DNA)是HBV的转录复制模板, 其大量制造的HBV病毒和HBsAg是导致感染者Anti-HBV免疫失能(immune dysfunction)进而造成HBV长期持续携带的重要原因之一。PEG-IFN/NAs两种药物虽能阻止病毒复制, 但并不能有效降低HBsAg水平, 更不能直接影响肝内cccDNA, 导致了多

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数患者即使在病毒复制被治疗长期控制的状态下,停药后仍然很快发生病毒学反弹(off-therapy viral relapse). 清除cccDNA是慢性乙肝治疗的终极目标,也是当前治疗新药研发的“圣杯”^[7]. 深入了解HBV生活史并在此基础上建立可靠的cccDNA检测方法对于靶向cccDNA乙肝新药的筛选和评估具有重要意义.

HBV感染肝细胞后,病毒脱衣壳露出松弛环状DNA(rcDNA)进入肝细胞核内修复完整双链并发生构象转变成超螺旋结构的cccDNA. cccDNA在肝细胞核内积聚,形成cccDNA池,然后以cccDNA作为模板,转录出病毒的各种RNA,制造病毒蛋白,完成DNA复制并组装形成新的子代病毒^[8]. 通常情况下,感染细胞内以cccDNA形式存在的病毒核酸水平和占比均不高,且cccDNA与其他形式的HBV DNA在序列组成上无差异,实现其特异性检测存在困难,以致在患者的肝组织和目前大多数体外和体内HBV感染模型中不易被检测^[9,10]. 因此,目前对cccDNA的形成、维持和降解机制仍知之甚少^[7,9,11].

目前cccDNA检测方法较为公认的方法是Southern blot,该方法基于核酸分子凝胶电泳迁移率差异,能够较好地地区分rcDNA(relaxed circular DNA)、线性dsDNA(double-stranded DNA)和cccDNA等不同形式的HBV核酸,检测结果准确可靠,但是分析灵敏度较低,操作也比较复杂,不适合于高通量的药物筛选和临床应用. 近年来,基于PCR扩增的新型核酸检测技术开始在cccDNA检测中进行应用探索,此类方法相对简便、分析灵敏度高,但易受到其他形式的HBV DNA的干扰,学界对其检测结果的准确性仍存疑虑. 本文通过对现有cccDNA检测方法研究的现状和进展进行系统总结,希望为发展简便易行且准确可靠的高灵敏度cccDNA定量检测方法提供思路和线索,进而促进靶向cccDNA的乙肝诊疗新技术发展.

1 HBV生活史

HBV感染的生活史主要包括下述过程(图1): (1) HBV通过其外膜蛋白与细胞膜上的HSPG和病毒受体NTCP吸附至肝细胞,病毒外膜与细胞膜融合后脱去外膜,核衣壳进入细胞内; (2) 核衣壳入核后rcDNA解脱负链5'端连接的末端蛋白、正链5'端连接的RNA残段^[12]; 以内源性DNA聚合酶延长正链,将各链的缺口封闭,最后转换成紧密的超螺旋cccDNA; (3) cccDNA作为模板转录出所有不同长度的HBV RNA (pgRNA、

pcRNA、S/PreS RNA、X RNA),进而翻译所有病毒蛋白,包括HBV核心抗原(HBcAg)、HBV e抗原(HBeAg)、HBV聚合酶(POL)、HBV表面抗原(HBsAg)和X蛋白(HBx). HBV通过反转录前基因组RNA(pgRNA)复制其基因组rcDNA,由此产生的rcDNA可被进一步包裹并分泌或再循环到细胞核中,以扩增cccDNA池^[13-16]. 除了来自病毒的rcDNA和胞内扩增途径外,cccDNA还可能通过双链线性DNA(double-stranded linear DNA, dslDNA)的非同源末端连接产生,但经此途径生成的cccDNA普遍存在缺陷^[12,17].

与cccDNA相比,rcDNA在正链与负链上均有缺口,只是部分区域互补故能形成环状但非超螺旋结构. 由于缺口的存在,rcDNA可以被一些核酸酶如外切核酸酶Ⅲ、绿豆核酸酶等降解成寡核苷酸或单核苷酸,而cccDNA由于是超螺旋且双链结构均完整,一般不会被上述酶降解. 研究上可利用cccDNA与rcDNA结构特征和特定核酸酶敏感性上的差异分离和检测cccDNA,如利用二氧化硅基树脂吸附柱纯化的Protein-free DNA结合外切酶(T5^[18]、Exonuclease V^[19])等方法可较好去除其他形式的HBV DNA.

2 cccDNA检测样品的提取制备方法

样品制备是准确检测cccDNA的关键环节. 核内cccDNA可以游离和浓缩的cccDNA核酸蛋白复合小体两种形式存在,提取cccDNA时一般需用十二烷基硫酸钠(SDS)、蛋白酶K或硫氰酸胍,β-巯基乙醇等裂解细胞核,释放DNA并去除蛋白质,然后丢弃细胞碎片,用酚氯仿进一步萃取上清并用乙醇沉淀法获得DNA^[10,20-25]. 另外,试剂盒QIAamp® DNA Blood & Tissue Mini Kit (QIAGEN, 德国)和 NucleoSpin® Tissue Kit (Macherey-Nagel, 德国)也被用来提取血清或组织细胞中的HBV DNA^[26-29],但是这两种试剂盒方法提取到的HBV DNA组成及比例尚未经过Southern blot验证,其与经典方法的效率差异也尚待系统比较. 1967年, Hirt等人^[30]提出了一种纯化染色体外DNA的方法,即在中性pH条件下用SDS裂解、盐沉淀处理细胞,再用酚氯仿萃取上清液,经此方法提取的DNA样品被称为“Hirt DNA”. Hirt DNA提取方法广泛应用于cccDNA检测,并被陆续改良^[13,15,31]. 尤其酚氯仿萃取步骤,可采用二氧化硅基树脂吸附结合去蛋白液清洗的离心柱法替代^[32],以简化操作步骤. 除了Hirt DNA提取法外,也有报道采用改良碱裂解法提取cccDNA的方法^[33-37].

图1 HBV生活史
Figure 1 Life cycle of HBV

作为示例，我们用撤去四环素并培养至第八天的HepAD38作为测试细胞样品，比较分析三种较为常用的HBV DNA提取方法(Total DNA, Core DNA及Hirt DNA，具体步骤参见图2注释)用于Southern blot检测HBV不同复制中间体及cccDNA的模式，结果如图2所示。其中图2(a)是Total DNA和Core DNA提取方法检测细胞内不同复制中间体的情况(地高辛标记探针检测)，可明显区分出ssDNA, dsDNA和rcDNA。图2(b)显示采用改良Hirt法结合二氧化硅基树脂吸附柱纯化的Protein-free DNA样品的杂交检测模型。Hirt DNA样品未经处理(图2(b), Untreated泳道)直接检测可显出rcDNA,

dsDNA和cccDNA。95°C热变性处理后，dsDNA和rcDNA转变成ssDNA。cccDNA变性后可快速复性，因此在95°C热变性处理后再用EcoR I酶切处理时呈dsDNA状态，可与其他形式的DNA有效区分。除了上述热变性联合内切酶的方法外，也可采用Plasmid-Safe ATP-Dependent DNase (PSAD)或T5外切酶等核酸酶来选择性降解Hirt DNA样品中的非cccDNA形式的DNA^[18,38,39]，当然它们并不能完全清除rcDNA对检测的干扰^[14,40]。2019年，Gao等人^[19]发现提取后的HBV DNA样品经过80°C变性后加入Exonuclease V 37°C反应30 min可有效去除其他非cccDNA形式的干扰，经

图 2 3种不同DNA提取方法对HepAD38细胞样品的Southern blot检测结果示例。Total DNA: 收集后的HepAD38细胞加入NET缓冲液(50 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 10 mmol/L NaCl, 0.5% NP-40)裂解后离心弃沉淀, 上清加入微球菌核酸酶和CaCl₂共同孵育后加入EDTA, 反应完全后, 再加入蛋白酶K和10% SDS消化和去除蛋白, 最后用酚氯仿抽提, 乙醇沉淀。Core DNA: 收集后的HepAD38细胞加入裂解缓冲液(10 mmol/L Tris-HCl pH 8.0, 0.1 mmol/L EDTA, 1% NP-40, 2%蔗糖)裂解后离心弃沉淀, 上清加入MgCl₂、RNase、DNase共同孵育后再加入HBV core沉淀缓冲液(35% PEG, 1.5 mol/L NaCl)沉淀弃上清, 接着加入Core消化缓冲液(0.5% SDS, 25 mmol/L TB8.0, 10 mmol/L EDTA, 150 mmol/L NaCl)和蛋白酶K消化去除蛋白, 而后用酚氯仿抽提, 乙醇沉淀。Hirt DNA: 收集后的HepAD38细胞依次加入缓冲液I(50 mmol/L Tris-HCl pH 7.5, 10 mmol/L EDTA)、缓冲液II(1.2% SDS)、缓冲液III(3 mol/L CsCl, 1 mol/L CH₃COOK, 0.67 mol/L CH₃COOH), 离心后上清转移至预平衡的柱子QIAGEN-tip 100洗脱

Figure 2 Examples of Southern blot assays using DNA specimens derived from HepAD38 cell lysates using three different extraction methods. For total DNA preparation: The HepAD38 cells were treated with NET buffer (50 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 10 mmol/L NaCl, 0.5% NP-40). After centrifuge, the pellets were discarded, and the supernatants were further treated with Micrococcal nuclease and CaCl₂, and then the EDTA was added to stop the reaction, following proteinase K digestion, phenol-chloroform deprotein extraction and ethanol precipitation. Core DNA: The cells were firstly treated with lysis buffer (10 mmol/L Tris-HCl pH 8.0, 0.1 mmol/L EDTA, 1% NP-40, 2% sucrose). The supernatants were collected for digestion with RNase and DNase (in presence of MgCl₂) and the core particles were subsequently precipitated with PEG8000 buffer. Subsequently, the precipitated core particles were further digested with proteinase K (in buffer with 0.5% SDS, 25 mmol/L TB8.0, 10 mmol/L EDTA, 150 mmol/L NaCl), following phenol-chloroform extraction and ethanol precipitation. For Hirt DNA preparation: the cells were firstly resuspended with Buffer I (50 mmol/L Tris-HCl pH 7.5, 10 mmol/L EDTA), following Buffer II (1.2% SDS) and Buffer III (3 mol/L CsCl, 1 mol/L CH₃COOK, 0.67 mol/L CH₃COOH). Finally, the lysis solution were centrifuged and loaded onto QIAGEN-tip 100 for extraction

Southern blot检测可得到特异性cccDNA条带, 相较于PSAD和T5外切酶, Exonuclease V处理的方法总体表现性能更佳。

实践表明, 上述提取方法中提取效率由高到低为: Total DNA, Core DNA, Hirt DNA。其中Total DNA和Core DNA提取方法提取临床组织样本时需注意去除蛋白干扰, 二者均能检测到胞内不同复制中间体的情况, 但无法较好区分cccDNA与其他形式的HBV DNA。Hirt DNA结合Exonuclease V处理方法可以检测到纯净的cccDNA, 但由于cccDNA在患者肝细胞内含量较少,

甚至检测不到, 且由于该方法提取效率较低, 因此需要较强的复制水平或更多的临床组织样本。

3 cccDNA的检测方法

除了Southern blot外, 已有多种基于核酸扩增的、灵敏度更高的方法被报道。总体上, cccDNA准确检测的核心问题是要避免其他形式的HBV DNA (尤其是rcDNA)的干扰。检测方法的选择很大程度上取决于待测样品中cccDNA的含量, 我们总结了2010~2019年间发表的有关慢性乙肝患者以及HBV感染模型动物(支

持HBV感染的嵌合小鼠)体内cccDNA水平的研究,归纳分析了不同研究检测的样品类型、检测方法以及测得cccDNA的水平(表1)。多数研究采用选择性qPCR来检测cccDNA拷贝数,而不同临床乙肝患者肝穿组织样品间cccDNA的水平有较大差异。为便于研读,我们将汇总于表1的数据根据不同研究检测样品的来源进行分类整理,获得表2。从表1和2的整理结果,可以看出在慢性乙肝患者中,HBsAg阳性且未治疗者其肝细胞内cccDNA拷贝数约在0.001~39.81拷贝/细胞(中位值为4.24),HBsAg阳性患者的经治后肝细胞内cccDNA拷贝数约为0.00219~7.57拷贝/细胞(中位值为3.786);HBsAg阴性未治疗者其肝细胞内cccDNA拷贝数约在0.0016~2.6拷贝/细胞(中位值为0.3),HBsAg阴性患者的经治后肝细胞内cccDNA拷贝数约为0.00158~0.4拷贝/细胞(中位值为0.25);隐匿性HBV感染者为0.00013~1.148拷贝/细胞(中位值为0.005);HBV感染的人肝/树鼯嵌合小鼠cccDNA拷贝数为1.0~4.6拷贝/细胞(中位值为1.2)(表2)。整体看来,HBsAg阳性患者的肝内cccDNA水平要高于HBsAg阴性患者;经治疗(NA或IFN)肝内cccDNA水平要低于未经治疗的患者,但仍可被测及;OBI感染者肝内和HBV感染相关HCC患者肝癌组织cccDNA水平大多处于极低水平。此外,HBV感染的嵌合小鼠(免疫缺陷)肝内cccDNA水平一般在每细胞几个拷贝,与典型的乙肝患者相当。值得注意的是,HBV感染肝细胞内cccDNA水平多仅为每细胞几个拷贝,整体水平大大低于血清HBV DNA水平,因此对于检测方法的灵敏度和精确度有较高的要求。

3.1 Southern blot

Southern blot是一种经典但复杂的cccDNA检测方法,它涉及探针制备、电泳、膜杂交和检测等几个过程^[66]。该方法是一种定性或相对定量的检测方法,将待测的DNA分子通过琼脂糖凝胶电泳进行分离,进而将其变性并按照其在凝胶中的位置转移至尼龙膜上,紫外交联固定后再与其他标记物的DNA探针进行反应,如果待测DNA中含有与探针互补的序列,则二者可以通过碱基互补原则,发生同源性杂交,再利用放射性自显影、化学发光或荧光成像检测。Southern blot检测使用的HBV特异性探针目前常使用³²P标记的同位素探针系统、地高辛或生物素等非同位素探针系统^[67,68]。相比而言,³²P标记探针检测法具有更低的背景,可通过延长放射性自显影的时间(可曝光数小时乃至数天不

等)大大提高灵敏度,但其安全性问题限制了其广泛使用。非同位素探针系统中,地高辛探针系统和生物素探针系统使用的最多,其优点是不存在³²P的放射性安全隐患,后期采用标记碱性磷酸酶或辣根过氧化物酶的Anti-DIG或Streptavidin结合化学发光显影成像,一般认为两种系统敏感性相当,但均低于使用³²P标记的同位素探针系统。根据实践经验,非同位素探针系统的主要问题在背景信号较强,往往难以长时间显影成像,影响了检测灵敏度。无论使用何种探针系统,使用Southern blot进行cccDNA检测均需要复杂耗时的操作过程,试剂成本较高,加上检测通量有限,难以实现自动化,限制了这一方法在药物筛选和临床实践中的应用。

3.2 基于PCR的cccDNA检测方法

3.2.1 常规选择性qPCR

1993年,Köck^[69]最早通过设计cccDNA特异性引物来检测cccDNA,此后,cccDNA的qPCR检测技术迅速发展。此方法一般采用跨缺口选择性PCR扩增,利用针对HBV负链缺口下游的TaqMan探针进行定量检测^[70]。然而,由于rcDNA与cccDNA的同源性,该方法对cccDNA的检测并非完全特异。近年来,研究者们通过不断对引物和探针进行优化,并改良实验方法,以提升该技术对cccDNA检测的特异性与灵敏度^[22,71,72]。2000年,Addison等人^[23]在Köck建立的PCR检测cccDNA技术的基础上发展了竞争性PCR方法。该方法通过已知浓度的竞争模板和未知浓度的目标模板以相同的扩增效率结合并竞争相同的cccDNA特异性引物后得到的扩增产物经琼脂糖凝胶电泳后进行Southern blot检测,根据竞争模板和目标模板的PCR产物长度和条带强度不同可进一步量化。该方法虽可提高cccDNA检测特异性与灵敏度,但是操作相对繁琐,不适合高通量检测应用。半巢式PCR技术检测cccDNA在1993年^[69]提出并经过改良^[73],该方法在HBV cccDNA定量中显示出较好的效果。与半巢式qPCR相似,巢式qPCR在2011年^[74]用于外周血单核细胞(PBMCs)和骨髓单核细胞(MMNCs)中的cccDNA进行定量研究^[28]。无论是巢式还是半巢式qPCR,由于引入了两轮的PCR扩增,其分析灵敏度大大提高,但是存在易污染造成假阳性,操作过程需格外谨慎,以防止产物污染。总之,选择性qPCR相比于Southern blot对cccDNA检测的简便性和灵敏度均有显著提高,还能实现定量检测,目前在多数实验室研究广泛被使用。其缺点是难以完全避免rcDNA带来的潜在干扰,

表 1 2010~2019年发表的涉及cccDNA定量水平的相关研究数据汇总分析^{a)}

Table 1 Summary of reported data regarding the levels of cccDNA in research papers published over 2010–2019

检测方法	人群/研究模型	样品来源	治疗	cccDNA (copies/cell)	参考文献
选择性qPCR	HBeAg(+)CHB (n=71)	肝组织	未经治疗	3.65 (1.43~8.55)	[41]
选择性qPCR	HBeAg(+)CHB (n=16)	肝组织	未经治疗	8.14 (6.2~10.7)	[42]
选择性qPCR	HBeAg(+)CHB (n=36)	肝组织	未经治疗	15.9 (0.1~141.8)	[43]
选择性qPCR	HBeAg(+)CHB (n=31)	肝组织	未经治疗	15.7 (1.0~137.2)	[43]
选择性qPCR	HBeAg(+)CHB (n=7)	肝组织/冷冻	未经治疗	0.32 (0.016~0.977)	[44]
选择性qPCR	HBeAg(+)CHB (n=28)	肝组织/冷冻	未经治疗	0.51 (0.0078~0.457)	[44]
选择性qPCR	HBeAg(+)CHB (n=30)	肝组织	未经治疗	1.6 (1.2~1.9)	[45]
选择性qPCR	HBeAg(+)CHB (n=36)	肝组织/冷冻	未经治疗	6.3 (1.4~18.1)	[46]
选择性qPCR	HBeAg(+)CHB (n=82)	肝组织	未经治疗	4.68 (0.851~25.70)	[47]
选择性qPCR	HBeAg(+)CHB (n=85)	肝组织/FFPE	未经治疗	28.84 (0.129~147.91)	[48]
选择性qPCR	HBeAg(+)CHB (n=16)	肝组织	未经治疗	8.14 (6.2~10.7)	[49]
选择性qPCR	HBeAg(+)CHB (n=16)	肝组织	PEG-IFN/48周	7.57 (3.5~9.5)	[49]
选择性qPCR	HBeAg(+)CHB (n=?)	ANTT	未经治疗	0.33 (0.0178~6.166)	[50]
选择性qPCR	HBeAg(+)CHB (n=30)	肝组织	未经治疗	1.43±0.979	[51]
选择性qPCR	HBeAg(+)CHB(n=27)	肝组织	未经治疗	3.8	[52]
选择性qPCR	HBeAg(+)CHB(n=85)	肝组织	未经治疗	20.89	[53]
选择性qPCR	HBsAg(+)CHB(n=28)	肿瘤	未经治疗	0.001	[54]
选择性qPCR	HBeAg(+)CHB(n=28)	非肿瘤肝组织	未经治疗	0.005	[54]
选择性qPCR	HBeAg(+)CHB(n=32)	肝组织	未经治疗	39.81(3.98~398.11)	[55]
ddPCR	HBeAg(+)CHB(n=14)	肝组织	ETV/>1年	0.00219	[56]
选择性qPCR	HBeAg(-)CHB(n=24)	肝组织	未经治疗	0.61 (0.1~1.3)	[42]
选择性qPCR	HBeAg(-)CHB(n=78)	肝组织	未经治疗	0.2 (0.1~0.43)	[41]
选择性qPCR	HBeAg(-)CHB(n=35)	肝组织	未经治疗	1.4 (0.02~114.5)	[43]
选择性qPCR	HBeAg(-)CHB(n=22)	肝组织	未经治疗	2.6 (0.02~213.0)	[43]
选择性qPCR	HBeAg(-)CHB(n=85)	肝组织/冷冻	未经治疗	0.0031 (0.00012~0.051)	[44]
选择性qPCR	HBeAg(-)CHB(n=39)	肝组织/冷冻	未经治疗	0.0016 (8.32×10 ⁻⁶ ~0.0257)	[44]
选择性qPCR	HBeAg(-)CHB(n=94)	肝组织/冷冻	未经治疗	0.09 (0.03~0.2)	[46]
选择性qPCR	HBeAg(-)CHB(n=25)	肝组织/FFPE	未经治疗	1.07 (0.1~7.079)	[48]
选择性qPCR	HBeAg(-)CHB(n=54)	肝组织	未经治疗	0.3 (0.1~1.1)	[57]
选择性qPCR	HBeAg(-)CHB(n=15)	肝组织	未经治疗	0.70 (0.18~1.93)	[57]
选择性qPCR	HBeAg(-)CHB(n=15)	肝组织	NAs/40.1±33.3月	0.40 (0.06~1.20)	[57]
选择性qPCR	HBeAg(-)CHB(n=24)	肝组织	未经治疗	0.61 (0.1~1.3)	[49]
选择性qPCR	HBeAg(-)CHB(n=24)	肝组织	PEG-IFN/48周	0.25 (0.0~0.9)	[49]
选择性qPCR	HBeAg(-)CHB(n=?)	ANTT	未经治疗	0.067 (0.00468~0.977)	[50]
选择性qPCR	HBeAg(-)CHB(n=30)	肝组织	未经治疗	6.58×10 ⁻² ±2.47×10 ⁻²	[51]
选择性qPCR	HBeAg(-)CHB(n=31)	肝组织	未经治疗	0.2	[52]
选择性qPCR	HBeAg(-)CHB(n=36)	肝组织	未经治疗	1.995	[55]
ddPCR	HBeAg(-)CHB(n=17)	肝组织	ETV/>1年	0.00158 (0.00123~0.0174)	[56]
选择性qPCR	HBsAg(-)OBI(n=25)	肝组织	未经治疗	1.148	[53]
选择性qPCR	HBsAg(-)OBI(n=40)	肝组织	未经治疗	0.005	[58]
ddPCR	HBsAg(-)OBI(n=27)	肝组织/冷冻	未经治疗	0.00013 (0.00005~0.00025)	[59]
选择性qPCR	HBeAg(+/-)CHB(44/18)	肝组织	NUC/2 年	0.115 (0.0288~0.457)	[47]

(续表1)

检测方法	人群/研究模型	样品来源	治疗	cccDNA (copies/cell)	参考文献
选择性qPCR	HBeAg(+/-)CHB(23/20)	肝组织/FFPE	未经治疗	7.28 (0.018~114.5)	[60]
选择性qPCR	HBeAg(+/-)CHB(23/20)	肝组织/FFPE	NUC/1 年	0.57 (<0.005~15.1)	[60]
选择性qPCR	HBeAg(未分类)CHB(n=56)	肝组织/FFPE	未经治疗	1.28 (0.15~3.22)	[20]
选择性qPCR	HBeAg(未分类)CHB(n=40)	肝组织	未经治疗	4.4 (0.016~137.2)	[58]
选择性qPCR	HBeAg(未分类)CHB(n=13)	肝组织	未经治疗	0.23 (0.02~2.54)	[61]
选择性qPCR	HBeAg(未分类)CHB(n=22)	肝组织	未经治疗	0.21 (0.01~1.47)	[61]
选择性qPCR	HBeAg(未分类)CHB(n=14)	肝组织	未经治疗	0.27 (0.01~0.48)	[61]
选择性qPCR	HBeAg(未分类)CHB(n=13)	肝组织	ETV/48周	0.06	[61]
选择性qPCR	HBeAg(未分类)CHB(n=22)	肝组织	ADV/48周	0.2	[61]
选择性qPCR	HBeAg(未分类)CHB(n=14)	肝组织	Placebo/48周	0.25	[61]
选择性qPCR	HBeAg(未分类)CHB(n=37)	肝组织/冷冻	未经治疗	1.0 (0.13~4.84)	[20]
选择性qPCR	HBeAg(未分类)HCC(n=23)	肝组织	未经治疗	<0.0002 (<0.0002~0.12)	[62]
选择性qPCR	HBeAg(未分类)CHB(n=13)	肝组织	未经治疗	0.0052 (<0.0002~0.12)	[62]
选择性qPCR	HBeAg(未分类)CHB(n=40)	肝组织	未经治疗	4.4	[58]
选择性qPCR	HBV感染的PHH小鼠	uPA/SCID Hu小鼠	未经治疗	1.0 (0.3~2.0)	[63]
选择性qPCR	HBV感染的PTH小鼠	uPA/SCID Tu小鼠	未经治疗	2.6	[64]
选择性qPCR	HBV感染的PHH小鼠	uPA/SCID Hu小鼠	未经治疗	0.3	[65]
选择性qPCR	HBV感染的PHH小鼠	uPA/SCID Hu小鼠	未经治疗	1.2	[65]
选择性qPCR	HBV感染的PHH小鼠	uPA/SCID Hu小鼠	未经治疗	4.6	[65]

a) CHB: 慢性乙肝(chronic hepatitis B); OBI: 隐匿性乙肝(occult hepatitis B); HCC: 肝细胞癌(hepatocellular carcinoma); FFPE: 福尔马林固定石蜡包埋(formalin fixed paraffin embedding); ANTT: 邻近非肿瘤组织(adjacent non-tumor tissues); PEG-IFN: 聚乙二醇干扰素(pegylated interferon alpha); ETV: 恩替卡韦(entecavir); ADV: 阿德福韦(adeфовir); NUC: 核苷(nucleos(t)ide); NA: 核苷类似物(nucleos(t)ide analogue); 未分类: 根据文献无法确定HBeAg阳性或者阴性

表 2 2010~2019年cccDNA检测拷贝数^{a)}

Table 2 A summary of copy number data of cccDNA reported in researches published over 2010~2019

人群/研究模型	治疗	cccDNA(拷贝/细胞)
HBeAg(+) CHB	否	4.24 (0.001~39.81)
	是	3.786 (0.00219~7.57)
HBeAg(-) CHB	否	0.3 (0.0016~2.6)
	是	0.25 (0.00158~0.4)
HBeAg(未分类) CHB	否	0.635 (0.0052~4.4)
	是	0.2 (0.06~0.25)
OBI	否	0.005 (0.00013~1.148)
HBV(+) HCC	否	<0.0002 (<0.0002~0.12)
HBV感染的嵌合小鼠	否	1.2 (1~4.6)

a) CHB: 慢性乙肝(chronic hepatitis B); OBI: 隐匿性乙肝(occult hepatitis B); HCC: 肝细胞癌(hepatocellular carcinoma); cccDNA(拷贝/细胞)计算: 括号前数值为表1中位数, 括号内数值为最小值与最大值

尤其是样品中rcDNA水平特别高时, 因此应该设置充分对照, 并与Southern blot结果对比验证, 否则常难以

被完全认可. 此外, 不同实验室采用的检测引物、探针及实验程序不完全相同, 缺乏统一标准, 常存在差异.

3.2.2 微滴-数字PCR

微滴-数字PCR(droplet-digital PCR, ddPCR)系统在近10年来迅速发展并开始用于cccDNA检测^[10,75-79]. 该方法通过将样本制备成数以万计的液滴, 每个液滴独立进行PCR反应. 含有可探测荧光信号的目标序列的液滴被标记为阳性事件, 如果液滴反应中没有信号, 则记录阴性事件. 该分析结果包含了数千乃至上万个反应, 构成一个泊松分布, 随后计算阳性和阴性微滴的数量, 从而以数字格式对cccDNA进行定量分析. 2018年, Huang等人^[80]采用基于探针的ddPCR方法可以实现慢乙肝患者血清, 单细胞和组织样本中cccDNA的检测, 且灵敏度和特异性均有出色表现, 大大提高了cccDNA的检测下限.

3.2.3 滚环扩增PCR和原位杂交PCR

在临床实践中, HBV cccDNA水平是判断乙肝病毒在肝脏复制状态的一个有价值的指标, 但其临床应用

受到采样和检测技术的严重制约。由于cccDNA在HBV DNA中所占的比例很小,血清中HBV cccDNA水平并不是敏感的病毒学指标^[20-22]。福尔马林固定石蜡包埋(FFPE)肝活检组织用于常规病理检查,比新鲜分离的肝活检组织更容易获得,与新鲜或冷冻保存的肝活检组织相比,FFPE标本在临床上更有应用价值,并且有大量的存档资料可供研究。2010年,Takkenberg等人^[20]首次报道用FFPE肝活检组织检测HBV cccDNA,显示肝内cccDNA的含量比冷冻保存的肝组织低100倍。这表明常规qPCR的灵敏度已经不能满足病理学家的需要。因此,Zhong等人^[27]设计了滚环扩增(RCA)与实时PCR相结合的方法解决了这一问题。该研究利用Phi29 DNA聚合酶设计了4组针对多个结合位点的RCA引物,由于phi29 DNA聚合酶在RCA中选择性地扩增了环状模板分子^[81],这一步骤的加入显著提高了HBV cccDNA检测的敏感性,获得了更高效的扩增效果,并且将经典方法中忽略的整合HBV DNA的影响降到最低。2014年,同一研究组将RCA与原位PCR(*in situ* PCR, IS-PCR)相结合并应用于检测cccDNA在FFPE肝组织中分布^[82]。IS-PCR是PCR和原位杂交(*in situ* Hybridization, ISH)的结合,最早由Haase等人^[83]在1990年提出。IS-PCR兼具PCR和ISH的优点,并被用于肝脏组织切片中病毒基因组或复制中间体的原位扩增^[84,85],可在单个细胞水平定位单个基因拷贝,但其敏感性和特异性仍然是应用的主要挑战。1998年,ISH首次被报道用于cccDNA的检测,它使用的是地高辛标记的单链探针检测可产HBV的HepG2细胞,肝组织的中cccDNA未被检测^[86],该方法可以满足低含量核酸检测所需的灵敏度和特异性。

RCA联合IS-PCR具有二者的优点,能敏感、特异地检测感染肝细胞中的HBV cccDNA,在光镜下可观察到HBV cccDNA在肝组织中的分布和定位,反映cccDNA表达水平与肝组织病理特征的关系,以评价抗HBV治疗效果。

最近的技术发展为在特别制备的组织样本和细胞培养中可视化检测cccDNA提供了新的可能性,其中包括cccDNA的荧光原位杂交(fluorescent *in situ* hybridization, FISH)检测^[87]。该方法首次揭示了鸭乙型肝炎病毒cccDNA和核HBV DNA在模拟抗病毒治疗条件下的命运。然而,cccDNA的可视化目前仍然是不切实际的,因为样品制备困难,需要复杂的设备来在临床环境中监测cccDNA。

3.2.4 入侵检测法

2004年,Wong等人^[88]首次利用入侵检测法测定乙肝患者血清中cccDNA含量。入侵检测法是一种用于基因分型和基因表达监测的非PCR信号扩增方法^[89,90],其主要原理如图3所示:该方法需要两个寡核苷酸、主探针和入侵者探针、以及一个荧光共振能量转移(FRET)盒。我们可利用cccDNA与其他形式HBV DNA的差异,设计主探针和入侵探针序列,进而得到cccDNA的阳性信号和非cccDNA的阴性信号。入侵检测法检测的特异性比较大,但是灵敏度较低,因此提取的DNA需要进一步的处理以消除干扰。

3.2.5 捕获杂交PCR

2015年首次在特定cccDNA捕获中引入磁性纳米颗粒,通过变性释放捕获的cccDNA,并对传统的qPCR进一步加工^[91]。该方法设计了特异性捕获HBV cccDNA的功能化纳米颗粒,该颗粒由链霉亲和素修饰,然后与生物素标记cccDNA特异性寡核苷酸偶联。在检测前对磁性纳米颗粒-链霉亲和素-生物素-cccDNA探针复合物进行了优化,并证明其能与cccDNA特异杂交。与直接实时荧光定量PCR相比,对于磁性捕获杂交qPCR(magnetic capture hybridization qPCR, MCH-qPCR)方法,高含量的HBV DNA不影响cccDNA的检测,cccDNA与rcDNA之间无交叉反应性。新型MCH-qPCR方法具有良好的灵敏度和特异性,可满足临床常规检测要求。但是由于该方法所需要的材料成本高,限制了其在针对cccDNA的高通量药物筛选中的应用。

4 与cccDNA高度相关的替代检测指标

无论采用何种方法,在临床实践和高通量药物筛选中直接进行cccDNA动态监测目前尚难以实现,促使了研究者探索和发展其他与cccDNA高度相关的替代检测指标。近10年以来,不断有研究者评估验证与cccDNA水平相关的HBV血清抗原和核酸指标。Zoulim^[92]研究发现,在抗病毒治疗中,肝组织活检样本中cccDNA的下降与血清HBsAg定量水平的下降高度相关,提示血清HBsAg的定量可能是肝内cccDNA含量的无创替代标志。2010年以后,HBsAg定量检测已在临床实践中广泛应用,其量化水平相对公认可作为对肝内cccDNA水平替代反映指标,当然由于HBsAg阴性的隐匿性HBV感染者肝内也可能检测到cccDNA,同时整合到宿主染色体中的病毒基因也可能转录翻译出HBsAg,两者的匹配度并不完全理想。在细胞模型中,Zhou等

图3 入侵检测法原理示意图

Figure 3 Schematic diagram of the invader assay for cccDNA detection

表3 HBsAg与感染者肝内cccDNA的相关性(根据2010~2019年发表的相关研究整理汇总)

Table 3 Correlation between HBsAg and intrahepatic cccDNA in patients with chronic HBV infection (a summary based on data of researches published over 2010–2019)

人群	治疗	替代指标 样品来源	cccDNA 样品来源	<i>r</i>	参考文献
合计		0.518 (0.322–0.811)			
HBsAg (+)	CHB(<i>n</i> =30)	未经治疗	血清	肝组织	<i>r</i> =0.517; <i>P</i> =0.008 [96]
	CHB(<i>n</i> =30)	PEG-IFN-α-2b/24月	血清	肝组织	<i>r</i> =0.673; <i>P</i> <0.001 [96]
	CHB(<i>n</i> =30)	降低	血清	肝组织	<i>r</i> =0.421; <i>P</i> =0.032 [96]
	HCC(<i>n</i> =89)	未经治疗	血清	肝组织	<i>r</i> =0.500; <i>P</i> <0.001 [97]
	HCC(<i>n</i> =89)	未经治疗	血清	肿瘤	<i>r</i> =0.280; <i>P</i> =0.01 [97]
	CHB(<i>n</i> =22)	未经治疗	血清	肝组织	<i>r</i> =0.470; <i>P</i> =0.03 [98]
	CHB(<i>n</i> =32)	PEG-IFN/48周	血清	肝组织	<i>r</i> =0.544; <i>P</i> =0.001 [55]
	CHB(<i>n</i> =32)	未经治疗	血清	肝组织	<i>r</i> =0.452; <i>P</i> =0.009 [55]
	CHB(<i>n</i> =52)	未经治疗/IC phase	血清	肝组织	<i>r</i> =0.652; <i>P</i> <0.001 [99]
	CHB(<i>n</i> =85)	未经治疗	血清	肝组织	<i>r</i> =0.710; <i>P</i> <0.001 [99]
	CHB(<i>n</i> =30)	未经治疗/IT phase	血清	肝组织	<i>r</i> =0.811; <i>P</i> <0.001 [99]
HBsAg (–)	CHB(<i>n</i> =26)	未经治疗	血清	肝组织	<i>r</i> =0.660; <i>P</i> <0.01 [98]
	CHB(<i>n</i> =25)	未经治疗	血清	肝组织	<i>r</i> =0.579; <i>P</i> =0.002 [99]
	CHB(<i>n</i> =59)	未经治疗	血清	肝组织	<i>r</i> =0.322; <i>P</i> =0.017 [100]
	CHB(<i>n</i> =46)	PEG-IFN/48周	血清	肝组织	<i>r</i> =0.364; <i>P</i> =0.029 [55]
HBsAg (未确定)	CHB(77/61)	治疗/未经治疗(124/14)	血清	肝组织	<i>r</i> =0.400; <i>P</i> <0.001 [101]
	CHB(24/12)	未经治疗	血清	肝组织	<i>r</i> =0.519; <i>P</i> =0.001 [102]
	CHB(111/28)	未经治疗	血清	肝组织	<i>r</i> =0.742; <i>P</i> <0.001 [99]

人^[31]的研究结果表明, HepAD38细胞中分泌的HBeAg与cccDNA有高度相关, 细胞培养上清液中HBeAg水平与细胞核内cccDNA的数量呈正相关. 因此, 以HBV 1.1倍基因组整合细胞为模型抗HBV药物筛选评估中, 培养上清中的HBeAg水平可作为胞内cccDNA水平和活

性的替代检测指标. 2012年, Cai等人^[93]通过检测上清液中的HBeAg, 在HepDE19细胞系中筛选小分化文库, 发现了两个新的能抑制cccDNA合成的抑制剂. 以HBeAg作为细胞模型中cccDNA替代检测指标的主要局限在于现有的HBeAg检测试剂所使用的检测抗体

表 4 HBeAg与感染者肝内cccDNA的相关性(根据2010~2019年发表的相关研究整理汇总)

Table 4 Correlation between HBeAg and intrahepatic cccDNA in patients with chronic HBV infection (a summary based on data of researches published over 2010–2019)

人群	治疗	替代指标 样品来源	cccDNA 样品来源	<i>r</i>	参考文献
合计				0.570 (0.507~0.678)	
HBeAg(+)	CHB(<i>n</i> =30)	未经治疗	血清	肝组织	<i>r</i> =0.507; <i>P</i> =0.010 [96]
	CHB(<i>n</i> =30)	PEG-IFN- α -2b/24月	血清	肝组织	<i>r</i> =0.678; <i>P</i> <0.001 [96]
	CHB(<i>n</i> =30)	未知	血清	肝组织	<i>r</i> =0.570; <i>P</i> =0.002 [96]

表 5 HBcrAg与感染者肝内cccDNA的相关性(根据2010~2019年发表的相关研究整理汇总)

Table 5 Correlation between HBcrAg and intrahepatic cccDNA in patients with chronic HBV infection (a summary based on data of researches published over 2010–2019)

人群	治疗	替代指标 样品来源	cccDNA 样品来源	<i>r</i>	参考文献
合计				0.64(0.323~0.929)	
HBeAg(+)	CHB(<i>n</i> =59)	未经治疗/IT phase	血清	肝组织	<i>r</i> =0.852; <i>P</i> <0.0001 [99]
	CHB(<i>n</i> =52)	未经治疗/IC phase	血清	肝组织	<i>r</i> =0.790; <i>P</i> <0.0001 [99]
	CHB(<i>n</i> =82)	未经治疗	血清	肝组织	<i>r</i> =0.323; <i>P</i> =0.004 [103]
	CHB(<i>n</i> =82)	NAs/96周	血清	肝组织	<i>r</i> =0.656; <i>P</i> =0.003 [103]
	CHB(<i>n</i> =85)	未经治疗	血清	肝组织	<i>r</i> =0.843; <i>P</i> <0.001 [48]
HBeAg(–)	CHB(<i>n</i> =18)	未经治疗/LR phase	血清	肝组织	<i>r</i> =0.730; <i>P</i> =0.0001 [99]
	CHB(<i>n</i> =10)	未经治疗/Reactivation	血清	肝组织	<i>r</i> =0.909; <i>P</i> <0.0001 [99]
	CHB(<i>n</i> =25)	未经治疗	血清	肝组织	<i>r</i> =0.865; <i>P</i> <0.001 [48]
HBeAg(未确定)	HCC(<i>n</i> =89)	未经治疗	血清	肝组织	<i>r</i> =0.436; <i>P</i> <0.001 [104]
	CHB(77/61)	治疗/未经治疗 (124/14)	血清	肝组织	<i>r</i> =0.700; <i>P</i> <0.001 [101]
	CHB(111/28)	未经治疗	血清	肝组织	<i>r</i> =0.929; <i>P</i> =0.0001 [99]
HBsAg(+)	CHB(<i>n</i> =57)	未经治疗	血清	肝组织	<i>r</i> =0.670; <i>P</i> <0.0001 [105]
	CHB(<i>n</i> =69)	NAs	血清	肝组织	<i>r</i> =0.380; <i>P</i> <0.0001 [105]
	CHB(<i>n</i> =126)	未经治疗/NA (57/69)	血清	肝组织	<i>r</i> =0.550; <i>P</i> <0.0001 [105]
	HCC(+)(<i>n</i> =51)	未知	血清	肝组织	<i>r</i> =0.600; <i>P</i> <0.0001 [105]
	HCC(–)(<i>n</i> =75)	未知	血清	肝组织	<i>r</i> =0.640; <i>P</i> <0.0001 [105]
FIB-4>1.653(<i>n</i> =63)		未知	血清	肝组织	<i>r</i> =0.470; <i>P</i> <0.0001 [105]
FIB-4<1.653(<i>n</i> =63)		未知	血清	肝组织	<i>r</i> =0.640; <i>P</i> <0.0001 [105]
F2纤维化(<i>n</i> =74)		未知	血清	肝组织	<i>r</i> =0.570; <i>P</i> <0.0001 [105]
F1纤维化(<i>n</i> =52)		未知	血清	肝组织	<i>r</i> =0.640; <i>P</i> <0.0001 [105]
A2/A3炎症(<i>n</i> =28)		未知	血清	肝组织	<i>r</i> =0.450; <i>P</i> <0.0001 [105]
A0/A1炎症(<i>n</i> =98)		未知	血清	肝组织	<i>r</i> =0.570; <i>P</i> <0.0001 [105]
HBV DNA<4 log IU/mL HCC (<i>n</i> =24)		未经治疗	血清	肝组织	<i>r</i> =0.408; <i>P</i> =0.001 [104]

往往存在与HBcAg的交叉反应性,易受到细胞分泌的无包膜HBcAg颗粒的干扰,而在外源启动子驱动的HBV 1.1倍基因组中HBcAg可由pgRNA直接翻译并不依赖于cccDNA。2016年,Cai等人^[94]通过将HA标签的编码序列引入HBV基因组中HBcAg起始密码子的5'端,构建了四环素控制型HBV稳定整合细胞株HepB-HAe82。在该细胞中,由于HA融合标签只存在于HBeAg中,可通过Anti-HA抗体对HBeAg进行精确辨别和定量检测,从而更好地反映细胞内cccDNA的水平。该方法部分解决了HBeAg的无干扰检测问题,局限在于需对病毒进行遗传改造,未来进一步发展针对HBeAg专有序列的高特异性抗体的发表可能成为解决该问题的途径。

除HBsAg和HBeAg外,近年来包括血清HBV DNA、HBcrAg(核心相关抗原)、HBV RNA等指标与感染者肝内cccDNA的相关性也被广泛研究。我们总结了2010~2019年间报道的血清替代指标与cccDNA相关性研究汇总于表3~6。整体看来,根据现有报道数据血清HBcrAg与cccDNA相关性相对更优(表5,相关系数 r

中位值约为0.640),HBeAg次之(表4,相关系数 r 中位值约为0.570),HBV DNA (r 中位值约为0.552)和HBsAg (r 中位值约为0.518)相关性相对较差。当然,HBeAg仅在部分患者中可被测及,而HBsAg在全部的慢性乙肝患者中均适用。血清HBV RNA是近年来报道的新指标,研究认为与肝细胞内cccDNA的转录活性有关,在评估抗HBV治疗停药后复发风险中具有临床价值^[95],但其与cccDNA的实际相关性尚待进一步研究。

5 讨论与小结

cccDNA是造成HBV感染长期持续、抗病毒治疗停药后病毒反弹的重要原因,从肝细胞中消除cccDNA被认为是治愈慢性HBV感染的关键^[7]。简单、高效、准确的cccDNA检测技术一直以来均是HBV研究领域重点关注的关键工具。尽管不断有新型cccDNA检测技术被报道,但除Southern blot外,多数新技术暂未能获得学界广泛认可。从方法学上看,cccDNA检测主要包括样品制备和检测两大步骤。在cccDNA样品制备过程中,其关键在于尽可能去除蛋白质和其他形式的HBV

表 6 HBV DNA与感染者肝内cccDNA的相关性(根据2010~2019年发表的相关研究整理汇总)^{a)}

Table 6 Correlation between HBV DNA and intrahepatic cccDNA of in patients with chronic HBV infection (a summary based on data of researches published over 2010–2019)

人群	治疗	替代指标 样品来源	cccDNA 样品来源	r	参考文献
合计				0.552(0.356–0.812) ^{b)}	
HBeAg(+)	CHB($n=85$)	未经治疗	血清	肝组织	$r=0.507$; $P<0.001$ [48]
	CHB($n=54$)	未经治疗	肝组织	肝组织	$r=0.736$; $P<0.00001$ [57]
HBeAg(–)	CHB($n=54$)	未经治疗	血清	肝组织	$r=0.356$; $P=0.012$ [57]
	CHB($n=18$)	未经治疗	肝组织	肝组织	$r=0.577$; $P<0.01$ [106]
	CHB($n=59$)	未经治疗	血清	肝组织	$r=0.506$; $P<0.001$ [100]
HBeAg (未知)	CHB(26/30)	未经治疗	血浆	肝组织	$r=0.648$; $P<0.0001$ [20]
	CHB(26/30)	未经治疗	血浆	肝组织	$r=0.812$; $P<0.0001$ [20]
	HCC($n=89$)	未知	肝组织	肝组织	$r=0.720$; $P<0.001$ [107]
	HCC($n=33$)	未知	血清	肝组织	$r=0.410$; $P=0.02$ [107]
	HCC($n=89$)	未经治疗	血清	肝组织	$r=0.549$; $P<0.001$ [104]
	CHB($n=20$)	未经治疗	肝组织	肝组织	$r=0.765$; $P<0.001$ [108]
	CHB(77/61)	治疗/未经治疗(124/14)	血清	肝组织	$r=0.700$; $P<0.0001$ [101]
HBsAg(+)	HCC($n=50$)	未经治疗	血清	肝组织	$r=0.552$; $P<0.001$ [109]
	HCC($n=106$)	未经治疗	血清	ANTT	$r=0.452$; $P<0.001$ [50]
HBV DNA>4 log IU/mL	HCC($n=65$)	未经治疗	血清	肝组织	$r=0.487$; $P<0.001$ [104]

a) CHB: 慢性乙肝(chronic hepatitis B); HCC: 肝细胞癌(hepatocellular carcinoma); ANTT: 邻近非肿瘤组织(adjacent non-tumor tissues); b) 括号前数值为 r 中位数, 括号内为最小值与最大值

DNA. Hirt DNA提取结合酶切处理相对有效,但尚需要进一步优化. 研究上需要制定相对标准化的操作程序,以便有效地比较不同的研究.

在cccDNA样品检测方法中, southern blot和选择性PCR是应用和改良最多的两种方法,前者虽不敏感且操作繁琐,但对cccDNA的区分度最佳,被广泛应用于

乙肝研究;基于选择性PCR的cccDNA检测方法未来发展的重点仍然是如何排除其他形式的DNA造成的干扰问题. 总之,乙肝领域的研究者应更加致力于技术发展,建立可靠的标准检测方法,跟踪或标记cccDNA,这将为Anti-HBV新药的发展和优化乙肝临床诊疗实践提供重要工具.

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Summary for “HBV cccDNA检测方法的研究进展”

Quantitative assays for covalently closed circular DNA of hepatitis B virus

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Chronic infection of hepatitis B virus (HBV) is still one of the public health concerns affecting human health. Although the prophylactic HBV vaccines was introduced over three decades ago, the total number of chronic hepatitis B (CHB) infection still accounts for 3.5% (257 million) of the world's population, with approximately 800000 annual deaths related to the viral infection. Currently, drugs including PEG-IFN and NAs are widely used in the treatment of HBV infection with certain degree of success. It is still difficult to achieve a high proportion of “clinical cure” with existing treatment methods. The presence of covalently closed circular DNA (cccDNA) in the infected hepatocytes is the underlying mechanism for the long-term HBV persistence and is also essential for off-therapy viral relapse. The current treatments have no or limited effects on the suppression of intrahepatic cccDNA. The availability for a reliable cccDNA quantification assay would facilitate the development of anti-viral drugs targeting cccDNA-targeting. In this review, we summarized the advantages and disadvantages of the existing methods for cccDNA quantitation. Specifically, the extraction method of Hirt DNA combined with Exonuclease V can effectively remove other forms of HBV DNA, and suitable for cccDNA extraction, but it needs to be further optimized. Southern blot is considered as the “gold-standard” method for cccDNA detection, but it is less sensitive and requires cumbersome procedures, making it an unlikely method for high-throughput screening used for drug development or for clinical applications. The PCR-based methods are more convenient and sensitive for cccDNA detection. However, the reliability of PCR-based method requires further optimization and validation. Different PCR-based methods were developed for quantitation of HBV cccDNA. They include selective qPCR, droplet-digital PCR, rolling circle amplification PCR, *in situ* PCR, invader assay, magnetic capture hybridization qPCR, etc. These PCR-based methods are widely used in clinical and laboratory research for quantitation of cccDNA. Efforts need to be made to minimize the interference from other forms of DNAs in the assays. More recently, hepatitis B core related antigen (HBcrAg) was found to be a novel marker of HBV, and its level in serum is highly correlated with intracellular cccDNA level. This biomarker, the HBcrAg level, can be used to indicate the suppression of cccDNA during different clinical antiviral therapies. The correlation needs further analysis with additional data sets. In summary, we present a review on the quantitation assays for HBV cccDNA. Such assays are important for drug developments and clinical applications for improved management of HBV infection related diseases. Performance and characteristics of various methods were compared with the areas of improvements for different assays.

HBV, cccDNA, anti-HBV drugs, quantitative cccDNA assay

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