

阵发性睡眠性血红蛋白尿症异常克隆特征研究

叶金慧 王璇 王世充 彭雪梅 葛美丽 邵英起 郑以州 程涛 胡林萍*

(中国医学科学院血液病医院(中国医学科学院血液学研究所), 细胞生态海河实验室, 实验血液学国家重点实验室, 国家血液系统疾病临床医学研究中心, 中国医学科学院&北京协和医学院, 天津 300020)

摘要 阵发性睡眠性血红蛋白尿症(PNH)是一类源于造血干细胞*PIGA*基因突变的获得性克隆性疾病, 临床表现主要为溶血、骨髓衰竭等。该文通过流式细胞术和体外集落形成等实验检测比较经典型PNH(cPNH)患者与健康对照组外周血细胞、骨髓中血细胞及造血干祖细胞(hemopoietic stem progenitor cells, HSPCs)的数量和功能, 探究PNH异常克隆特征。结果显示, cPNH患者外周血细胞中T细胞、B细胞和NK细胞数明显降低, 髓系细胞数无明显改变。骨髓各类血细胞中, cPNH患者单核细胞比例明显高于对照组, 中性粒细胞、有核红细胞以及淋系细胞比例无明显改变。cPNH患者骨髓HSPCs均为CD59⁻的PNH异常克隆; 患者HSPCs出现大量耗竭, 向B-NK祖细胞分化明显受阻, 该结果与外周血和骨髓中B、NK细胞变化一致, 提示cPNH患者淋系细胞受损最为严重。CFU实验结果表明, cPNH患者HSPCs形成髓系克隆的能力与健康对照组无差异; 而在红系克隆种类中, cPNH患者形成的偏小的红系克隆代偿性增多。综上, 该研究解析了PNH异常克隆的生物学特征, 为PNH的诊治提供了一定的理论依据。

关键词 阵发性睡眠性血红蛋白尿症; PNH克隆; 特征; 造血干祖细胞

The Biological Characteristics of PNH Clones

YE Jinhui, WANG Xuan, WANG Shichong, PENG Xuemei, GE Meili, SHAO Yingqi,
ZHENG Yizhou, CHENG Tao, HU Linping*

(Institute of Hematology & Blood Diseases Hospital, Haihe Laboratory of Cell Ecosystem,
State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases,
Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China)

Abstract PNH (paroxysmal nocturnal hemoglobinuria) is an acquired clonal disease caused by the *PIGA* mutation in HSCs (hematopoietic stem cells), characterized mainly by hemolysis and bone marrow failure etc. In this study, flow cytometry and colony forming cell assays were performed to analyze the total number of blood cells and HSPCs in bone marrow and peripheral blood from cPNH patients compared with health donors. Moreover, the characteristics of PNH clones were investigated. The results revealed that the number of T cells, B cells and NK cells in peripheral blood from cPNH decreased significantly, while no significant difference was observed in the number of myeloid cells compared with health donors. Among many kinds of blood cells in bone marrow, the pro-

收稿日期: 2022-05-04 接受日期: 2022-05-19

国家自然科学基金(批准号: 81970104、81890990)、细胞生态海河实验室创新项目(批准号: HH22KYZX0040)、国家重点研发计划(批准号: 2021YFA1100900)、中国医学科学院医学与健康科技创新工程项目(批准号: 2021-I2M-1-040)和中国医学科学院基金(批准号: 2021-RW320-011)资助的课题

*通讯作者。Tel: 022-23909166, E-mail: hulinping@ihcams.ac.cn

Received: May 4, 2022 Accepted: May 19, 2022

This work was supported by the National Natural Science Foundation of China (Grant No.81970104, 81890990), the Haihe Laboratory of Cell Ecosystem Innovation Fund (Grant No.HH22KYZX0040), the National Key R&D Program of China (Grant No.2021YFA1100900), the CAMS Innovation Fund for Medical Sciences (CIFMS) (Grant No.2021-I2M-1-040), and the Foundation of Chinese Academy of Medical Sciences (Grant No.2021-RW320-011)

*Corresponding author. Tel: +86-22-23909166, E-mail: hulinping@ihcams.ac.cn

portion of monocytes in cPNH was significantly higher than that in the control group, while there was no significant difference in the proportion of neutrophils, nucleated erythrocytes and lymphoid cells between cPNH and control group. All the HSPCs from cPNH bone marrow were abnormal PNH clones detected as CD59⁻. Moreover, there was an extensive exhaustion of HSPCs in cPNH, and their lymphoid differentiation potential was obviously inhibited, which was consistent with the change of B cells and NK cells in peripheral blood and bone marrow. These data indicated that lymphoid cells were the most severely impaired cells in cPNH. CFU assays manifested that there was no difference in myeloid colony forming ability between control and cPNH. As for the erythroid colony, cPNH HSPCs formed more small erythroid colonies for compensation. In conclusion, this study showed the biological characteristics of PNH clones, which provided theoretical basis for the diagnosis and treatment of PNH.

Keywords paroxysmal nocturnal hemoglobinuria; PNH clones; characteristics; hemopoietic stem progenitor cells

阵发性睡眠性血红蛋白尿症(paroxysmal nocturnal hemoglobinuria, PNH)是源于造血干细胞(hematopoietic stem cells, HSCs)的X连锁基因*PIGA*发生体细胞突变造成的获得性克隆性疾病^[1]。*PIGA*基因参与编码糖基磷脂酰肌醇(glycosyl phosphatidyl inositol, GPI)锚合成所需的糖基转移酶^[2], 人类蛋白中约有150种蛋白通过GPI锚连^[2-3]。PNH患者*PIGA*突变导致GPI缺失, 锚连蛋白不能通过GPI结合在细胞表面, 因此突变血细胞表现为CD55、CD59、CD14、CD16等GPI锚连蛋白部分或完全缺失。而CD55、CD59作为补体调节蛋白, 保护红细胞免受激活的补体系统攻击。在PNH中, 由于CD55、CD59的缺失, 患者表现出补体介导的血管内溶血及溶血带来的相关症状, 如贫血、骨髓衰竭、血红蛋白尿、肺动脉高压、肌张力障碍等^[4]。PNH属于罕见病, 在美国的发病率为每年每百万人中5.7例^[5], 在英国的发病率为每年每百万人中3.5例^[6]。PNH与获得性再生障碍性贫血(aplastic anemia, AA)关系密切, 有约70%的AA患者在确诊时伴有PNH克隆, 约40%的PNH由AA转化而来^[7-8]。2%~6%的PNH患者会在10年内继发骨髓增生异常综合征或急性髓系细胞白血病^[9]。因此, 深入研究PNH异常克隆生物学特征具有重要的临床意义。

近年来, 采用流式细胞术检测各类血细胞GPI锚连蛋白的缺失及PNH克隆大小, 已成为诊断PNH的“金标准”。早期红细胞PNH克隆主要通过流式检测CD59, 粒细胞PNH克隆主要通过检测CD55和CD59来判断^[10]。尽管早期的Ham实验、蔗糖溶血实验等辅助检查手段的敏感性已显著提高, 但在PNH克隆较低时, 检测结果仍不够精确和敏感^[11]。

荧光标记的嗜水气单胞菌溶素变异体(fluorochrome-conjugated mutant aerolysin toxin, FLAER)的应用进一步提高了PNH克隆检测的敏感性和特异性。FLAER是由Alexa 488标记的嗜水气单胞菌溶素前体的无活性变体, 可以和所有胞膜的GPI锚连蛋白特异性结合, 且不引起细胞裂解, 因此其能更精确地反映突变细胞GPI锚连蛋白的缺失情况^[12]。基于PNH克隆大小和其他临床检测, 2005年国际PNH工作组将该病分为经典型、合并其他骨髓衰竭性疾病和亚临床型三类^[13]。

随着PNH克隆检测方法的逐渐提升, 人们对其特征认识也不断加深。在PNH患者中, 经典型患者的PNH克隆平均大小超过70%; 在亚临床和伴骨髓衰竭的患者中, PNH克隆往往较经典型患者小^[14-15]; 在约1%的健康人群可以检测到0.02%~0.03%的PNH突变细胞群^[16-17], 而这类PNH克隆突变可能来源于造血祖细胞^[18]。PNH克隆是如何维持的呢? 特别是在正常造血细胞同时存在下如何扩增? 其机制仍未明了。可能的原因有以下两方面。一方面是在内源性因素的作用下突变HSCs中发生的一个或多个额外的遗传改变赋予该HSCs克隆生存和生长优势, 如*TET2*、*JAK2*、*ASXL1*、*BCOR*、*SUZ12*等一些参与髓系肿瘤发病或癌症克隆进化的关键驱动因素^[9,19-20]。另一方面是外源性因素介导的克隆选择, 如PNH克隆存活优势与免疫逃逸密切相关^[21]; PNH异常HSCs不易凋亡, 具有更强的增殖能力等^[22-24]。

本研究通过流式细胞术、体外集落形成实验等方法检测并比较经典型PNH(classic PNH, cPNH)患者与健康对照组外周血以及骨髓中的血细胞和造血细胞的数量及功能差异, 研究PNH异常克隆生物学

特征,为PNH的临床诊治提供理论基础。

1 材料和方法

1.1 材料

IMDM培养基、胎牛血清(fetal bovine serum, FBS)、青霉素/链霉素(penicillin/streptomycin, P/S)购于Gibco公司;H4034半固体培养基购于Stem Cell Technologies公司;人细胞因子Flt3-L、IL-6购于Pepro Tech公司;流式抗体人Lineage cocktail-APC、CD15-APC、CD64-APC、CD34-BV510、CD38-FITC、CD45RA-APC-Cy7、CD90-Percep-Cy5.5、CD45-APC-Cy7、CD15-BV510、CD19-PE、CD56-Percep-Cy5.5、CD3-BV605购于BioLegend公司;CD10-BV786、CD135-PE、CD59-BV605、Brilliant Stain Buffer购于BD公司;CD235a-PE、CD41-PE-Cy7购于Beckman Coulter公司;FLAER-Alexa 488购于Cedarlane公司;DAPI(4',6-diamidino-2-phenylindole)购于Sigma公司。

1.2 研究对象

该研究中使用的人细胞样本来自中国医学科学院血液病医院,患者均签署知情同意书。该研究由中国医学科学院血液病医院伦理委员会审批(批准号:KT2019092-EC-2)。经典型PNH诊断标准参考文献[13,25],基于2005年国际PNH工作组的临床分类^[13]和2018年《血液病诊断及疗效标准》(第4版)^[25],经典型PNH患者有典型的溶血和血栓形成。具体表现为LDH显著增高伴阵发性的血红蛋白尿;骨髓增生活跃伴红系造血旺盛或出现轻微形态异常;流式细胞检测显示GPI-中性粒细胞>50%。本研究纳入的经典型PNH患者其GPI-中性粒细胞在95%以上。《阵发性睡眠性血红蛋白尿症流式细胞术检测中国专家共识》(2021年版)^[26]和于2018年发表的国际临床流式协会(ICCS)/欧洲临床细胞分析协会(ESCCA)国际指南^[27]规范了PNH克隆的命名:(1)PNH细胞>1.0%时,为PNH克隆;(2)PNH细胞为0.1%~1.0%时,为低量PNH克隆;(3)PNH细胞<0.1%时,为存在少量GPI缺陷细胞,或存在少量PNH表型细胞。

1.3 样本处理

提前准备红细胞裂解液(ACK),将其平衡至室温,并将其加至无菌50 mL离心管中,将新鲜送样的骨髓或外周血轻柔吹匀,加入装有ACK的离心管中,充分混匀,冰上裂解10 min。后于4 °C、1 500 r/min离心5 min,弃尽上清。以10~20 mL PBE缓冲液充分

混匀,取20 μL并计数,其余离心弃尽上清。得到的细胞可用于流式检测、流式分选或冻存至液氮。

1.4 流式表面标记及分选

在每10⁷个细胞中分别添加每种抗体各2 μL,当使用多于2种BV系列抗体时添加10 μL/管Brilliant Stain Buffer。抗体选择参考课题组前期工作、国际指南^[11]和中国专家共识^[26]。检测人成熟血细胞的抗体为:CD45-APC-Cy7、CD15-BV510、CD64-APC、CD19-PE、CD56-Percep-Cy5.5、CD3-BV605、CD235a-PE、CD41-PE-Cy7、FLAER-Alexa 488、Brilliant Stain Buffer。检测人造血干祖细胞的抗体为:人Lineage cocktail(CD3、CD19、CD20、CD56)-APC、CD15-APC、CD64-APC、CD34-BV510、CD38-FITC、CD45RA-APC-Cy7、CD90-Percep-cy5.5、CD10-BV786、CD135-PE、CD59-BV605。FMO(fluorescence minus one)对照添加除FLAER或CD59外的全部抗体(稀释比例均为1:1 000);阴性对照不添加检测抗体。将抗体加入细胞悬液中,充分混匀,4 °C避光孵育30 min。加入2~3 mL PBE缓冲液洗涤,离心后弃上清,每10⁷细胞用1 mL PBE缓冲液重悬,并按1:1 000的比例加入DAPI上机分析或分选。

1.5 成熟血细胞表面标记及流式分群

根据细胞表面标记,我们将血细胞分为六类流式表型细胞。中性粒细胞(neutrophil, Neu):CD45⁺CD64⁻CD15⁺;单核细胞(monocyte, Mono):CD45⁺CD15⁻CD64⁺;B淋巴细胞:CD45⁺CD64⁻CD15⁻CD3⁻CD19⁺;T淋巴细胞:CD45⁺CD64⁻CD15⁻CD56⁻CD3⁺;自然杀伤细胞(natural killer cells, NK):CD45⁺CD64⁻CD15⁻CD19⁻CD3⁻CD56⁺;红细胞(erythrocyte, E):CD45⁻CD235a⁺。其中,中性粒细胞和单核细胞的和为髓系细胞(myeloid cell, My)。

1.6 骨髓中造血干细胞和各类造血祖细胞表面标记

根据细胞表面标记,我们将骨髓中的造血干祖细胞(Lin⁻CD34⁺)分为以下八类。(1)造血干细胞(HSCs):Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺;(2)多潜能祖细胞(multipotent progenitors, MPPs):Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁻;(3)共同髓系祖细胞(common myeloid progenitors, CMPs):Lin⁻CD34⁺CD38⁺CD10⁻CD45RA⁻CD135⁺;(4)巨核红系祖细胞(megakaryocyte-erythroid progenitors, MEPs):Lin⁻CD34⁺CD38⁺CD10⁻CD45RA⁻CD135⁻;(5)粒-单核系祖细胞(granulocyte-monocyte

progenitors, GMPs): $\text{Lin}^- \text{CD34}^+ \text{CD38}^+ \text{CD10}^- \text{CD45RA}^+ \text{CD135}^+$; (6) 淋系倾向多潜能祖细胞 (lymphoid-primed multipotent progenitors, LMPPs): $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD45RA}^+ \text{CD90}^- \text{CD10}^-$; (7) 淋系祖细胞 (multilymphoid progenitors, MLPs): $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD45RA}^+ \text{CD90}^- \text{CD10}^+$; (8) B-NK祖细胞 (B-NK progenitors, B-NKPs): $\text{Lin}^- \text{CD34}^+ \text{CD38}^+ \text{CD45RA}^+ \text{CD10}^+$.

1.7 集落形成(Colony forming unit, CFU)实验

在 H4034 甲基纤维素培养基中加入 1% P/S、50 ng/mL 人 IL-6、20 ng/mL 人 Flt3-L, 充分涡匀, 静置排出气泡, 分装并冻存于 -20°C 冰箱。实验前将其置于 4°C 融化。分别分选 3 例健康供者骨髓 $\text{Lin}^- \text{CD34}^+ \text{CD59}^+$ 细胞和 3 例经典型 PNH 患者骨髓 $\text{Lin}^- \text{CD34}^+ \text{CD59}^-$ 细胞, 收集于 300 μL 的 IMDM+10% FBS+1% P/S 中。将细胞加入 3 mL 半固体培养基中(600 个/管), 充分涡旋混匀后将细胞接种于 24 孔板中, 500 μL /孔, 每例样本做 5 个复孔, 24 孔板外用 PBS 液封。将其放入培养箱 37°C 、5% CO_2 培养 12~14 天, 在倒置显微镜下对形成的集落进行计数。集落种类包括: 红系爆式集落形成单位(burst forming unit-erythroid, BFU-E), 红系集落形成单位(colony forming unit-erythroid, CFU-E), 粒、巨噬细胞集落形成单位(colony forming unit-granulocyte, macrophage, CFU-GM), 粒、红、巨噬、巨核细胞集落形成单位(CFU-granulocyte, erythrocyte, macrophage, megakaryocyte, CFU-GEMM)。其中红

系克隆按大小分为三类: CFU-E、小 BFU-E (small BFU-E, 直径 $<500 \mu\text{m}$)、大 BFU-E (large BFU-E, 直径 $>500 \mu\text{m}$)。髓系克隆按单个克隆中的细胞数分为三类: 小 CFU-GM (small CFU-GM, 20~50 个细胞)、中等 CFU-GM (medium CFU-GM, 50~200 个细胞)、大 CFU-GM (large CFU-GM, >200 个细胞)。

1.8 数据分析

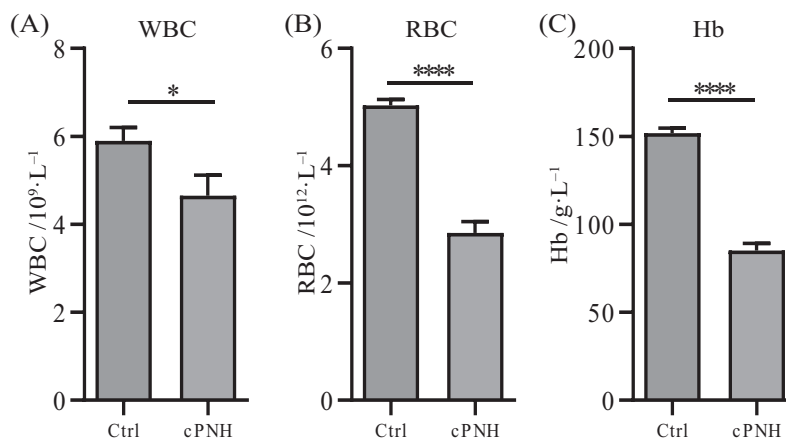
采用 GraphPad Prism 8.0 软件进行统计学分析和作图。流式细胞数据分析采用 FlowJo v10.0。实验数据以均值 \pm 标准差表示, 两组间数据比较采用 *t* 检验, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 cPNH 患者外周血改变

收集 18 位健康对照 (Ctrl) 和 21 位经典型 PNH 患者 (cPNH) 的血常规数据, 并进行统计。健康对照组的白细胞数为 $(5.90 \pm 0.30) \times 10^9/\text{L}$, 患者组为 $(4.65 \pm 0.47) \times 10^9/\text{L}$, 患者组较对照组明显降低 (图 1A, $P < 0.05$)。健康对照组红细胞数为 $(5.03 \pm 0.10) \times 10^{12}/\text{L}$, 患者组为 $(2.85 \pm 0.19) \times 10^{12}/\text{L}$, 患者组较对照组明显降低 (图 1B, $P < 0.0001$), 且其均值低于临床参考值 $(3.5 \sim 5.5) \times 10^{12}/\text{L}$ 。健康对照组血红蛋白为 $(151.89 \pm 2.86) \text{g/L}$, 患者组为 $(85.17 \pm 4.07) \text{g/L}$, 患者组较对照组明显降低 (图 1C, $P < 0.0001$), 且其低于临床参考值 $110 \sim 160 \text{g/L}$ 。

将 4 例健康对照和 12 例 cPNH 患者外周血流式结果纳入统计。流式检测结果显示, 健康对照组



A: 白细胞数目; B: 红细胞数目; C: 血红蛋白数目。* $P < 0.05$, **** $P < 0.0001$, 经典型PNH(cPNH)与健康对照组(Ctrl)相比。Ctrl: $n=18$; cPNH: $n=21$ 。

A: WBC (white blood cell) count; B: RBC (red blood cell) count; C: Hb (hemoglobin) count. * $P < 0.05$, **** $P < 0.0001$ cPNH vs Ctrl group. Ctrl: $n=18$; cPNH: $n=21$.

图1 经典型PNH血常规参数分析

Fig.1 Parametric statistics of complete blood count of cPNH

中性粒细胞和单核细胞中FLAER⁺群比例为100%,即未检测到PNH克隆(图2A);患者组中性粒细胞和单核细胞FLAER⁺群比例均在97%以上,即绝大多数细胞为PNH克隆(图2B)。对血细胞流式分群结果分析,相较于健康对照组,cPNH患者外周血细胞中,B细胞比例明显降低[图2C,cPNH(1.44%±0.20%) vs Ctrl(3.72%±0.68%),*P*<0.01],T细胞比例两者无明显差异。cPNH患者髓系细胞比例略有减少,其中单核细胞的比例明显升高[图2C,cPNH(9.83%±1.83%) vs Ctrl(4.27%±0.61%),*P*<0.01];中性粒细胞比例降低;有核红细胞比例无明显差异。相较于健康对照,cPNH患者T细胞、B细胞和NK细胞数均明显减少[图2D,T细

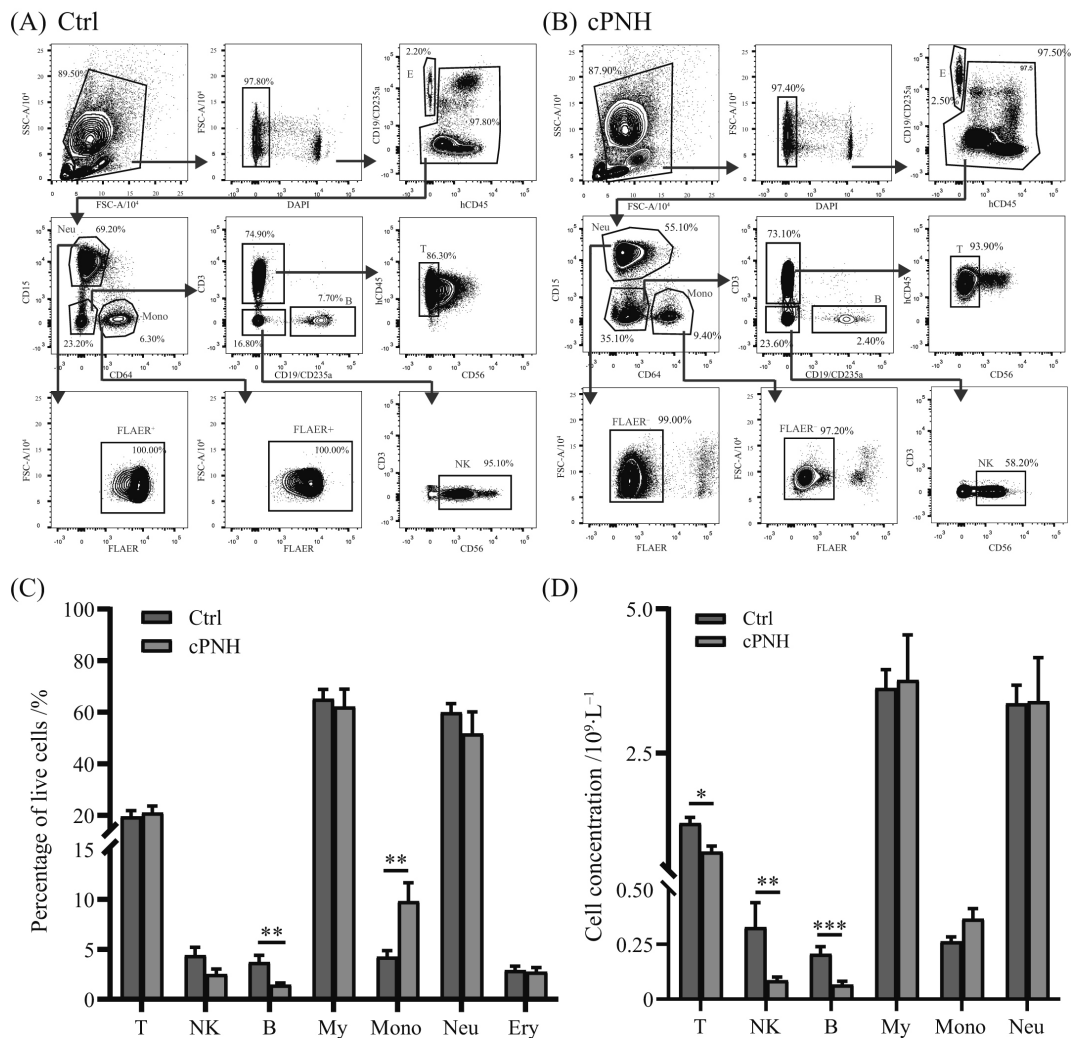
胞:cPNH(0.79±0.10)×10⁹/L vs Ctrl(1.28±0.10)×10⁹/L,*P*<0.05; B细胞:cPNH(0.07±0.02)×10⁹/L vs Ctrl(0.21±0.03)×10⁹/L,*P*<0.001; NK细胞:cPNH(0.09±0.02)×10⁹/L vs Ctrl(0.33±0.11)×10⁹/L,*P*<0.01]。髓系细胞(My)数无明显差异。

2.2 cPNH患者骨髓中血细胞改变

流式分析骨髓中各类血细胞的比例(图3A和图3B),结果显示,与健康对照组相比,cPNH患者髓系细胞中单核细胞比例明显高于对照组[图3C,cPNH(11.3%±2.1%) vs Ctrl(3.7%±0.7%),*P*<0.05]。中性粒细胞、有核红细胞以及淋系细胞比例变化无差异。

2.3 cPNH患者骨髓造血干祖细胞改变

根据细胞表面标记,我们将骨髓中的造血干



A、B: 健康对照组(Ctrl)和经典型PNH(cPNH)外周血(PB)中血细胞流式代表图; C: 外周血各类成熟细胞比例; D: 每毫升外周血中各类成熟细胞数。**P*<0.05, ***P*<0.01, ****P*<0.001, 经典型PNH与健康对照组相比。Ctrl: *n*=4; cPNH: *n*=12。

A,B: representative flow plots for blood cells from PB from a Ctrl and a cPNH case; C: the proportion of blood cells in PB; D: blood cell counts in per milliliter of PB. **P*<0.05, ***P*<0.01, ****P*<0.001 cPNH vs Ctrl group. Ctrl: *n*=4; cPNH: *n*=12.

图2 外周血中成熟血细胞分类及PNH克隆统计

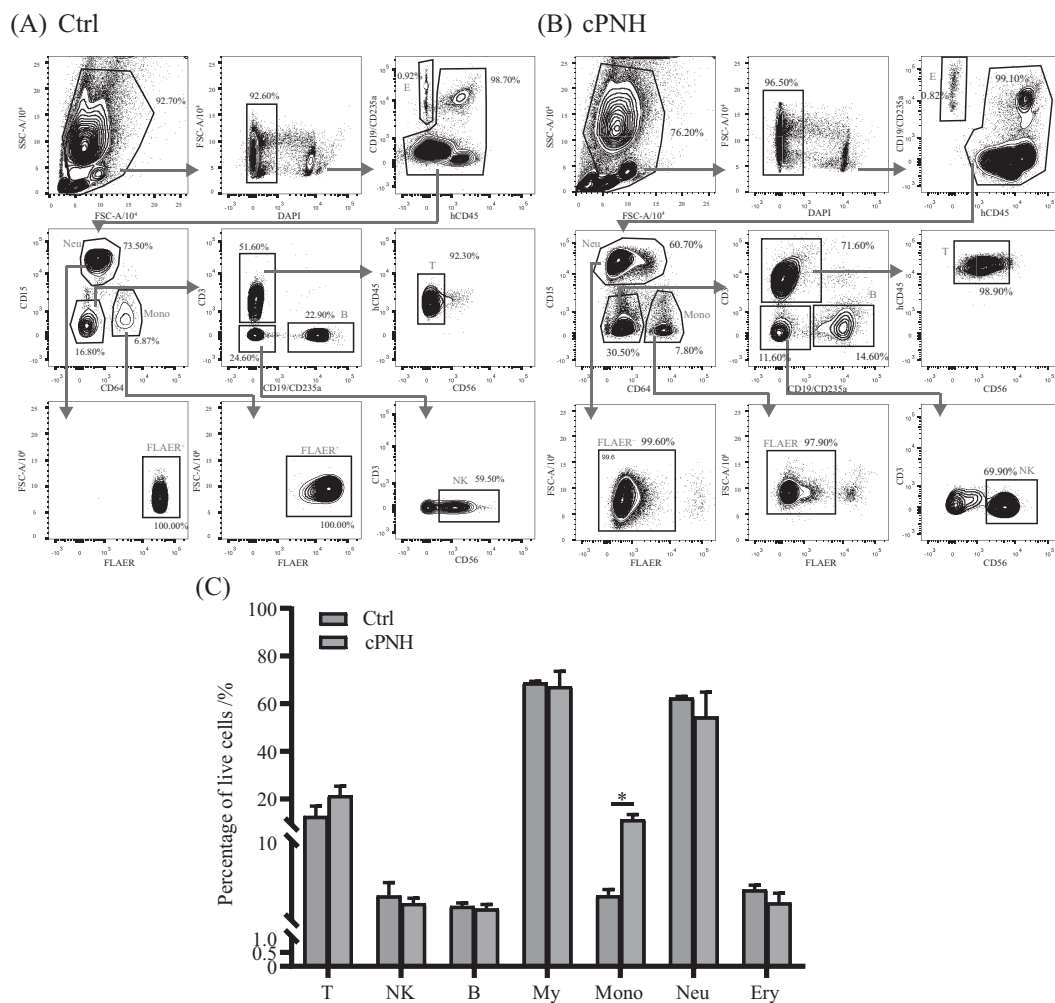
Fig.2 Statistics of mature cells and PNH clones in PB

祖细胞(Lin⁻CD34⁺)分为八类(图4A和图4B)。流式检测结果显示,健康对照组骨髓中的八类造血干祖细胞均为CD59⁺的细胞,即不存在PNH克隆;而cPNH患者组中八类造血干祖细胞CD59⁺的细胞比例为98.5%以上,即绝大部分均为PNH异常克隆(图4C)。流式分析cPNH患者骨髓中八类造血干祖细胞的比例,结果显示,与健康对照组相比,cPNH患者骨髓中Lin⁻CD34⁺CD38⁻细胞比例下降[cPNH(0.07%±0.01%) vs Ctrl(0.59%±0.11%), *P*<0.05];HSCs+MPPs细胞比例显著降低[cPNH(0.03%±0.01%) vs Ctrl(0.40%±0.08%), *P*<0.05]。骨髓各类祖细胞中,cPNH患者B-NKPs和MLPs的降低幅度最大,[B-NKPs: cPNH(0.006%±0.020%) vs Ctrl(0.207%±0.065%),

P<0.05; MLPs: cPNH(0.002%±0.001%) vs Ctrl(0.047%±0.019%), *P*<0.05];LMPPs、CMPs、GMPs和MEPs均降低(图4D)。以上结果提示,在cPNH患者中HSPCs比例明显减少,以淋系B-NKPs和MLPs受损最为严重。同时,CMPs、GMPs和MEPs在CD34⁺的HSPCs中的比例和对照组无差异,提示cPNH患者的HSCs向CMPs、GMPs和MEPs分化无影响,而B-NKPs在CD34⁺的HSPCs中的比例明显下降[cPNH(3.18%±0.85%) vs Ctrl(10.65%±2.14%), *P*<0.05],说明HSCs向B-NKPs分化受阻(图4E)。

2.4 cPNH患者HSPCs体外集落形成能力

对健康对照组HSPCs(Lin⁻CD34⁺CD59⁺)和cPNH患者的HSPCs(Lin⁻CD34⁺CD59⁻)进行CFU实



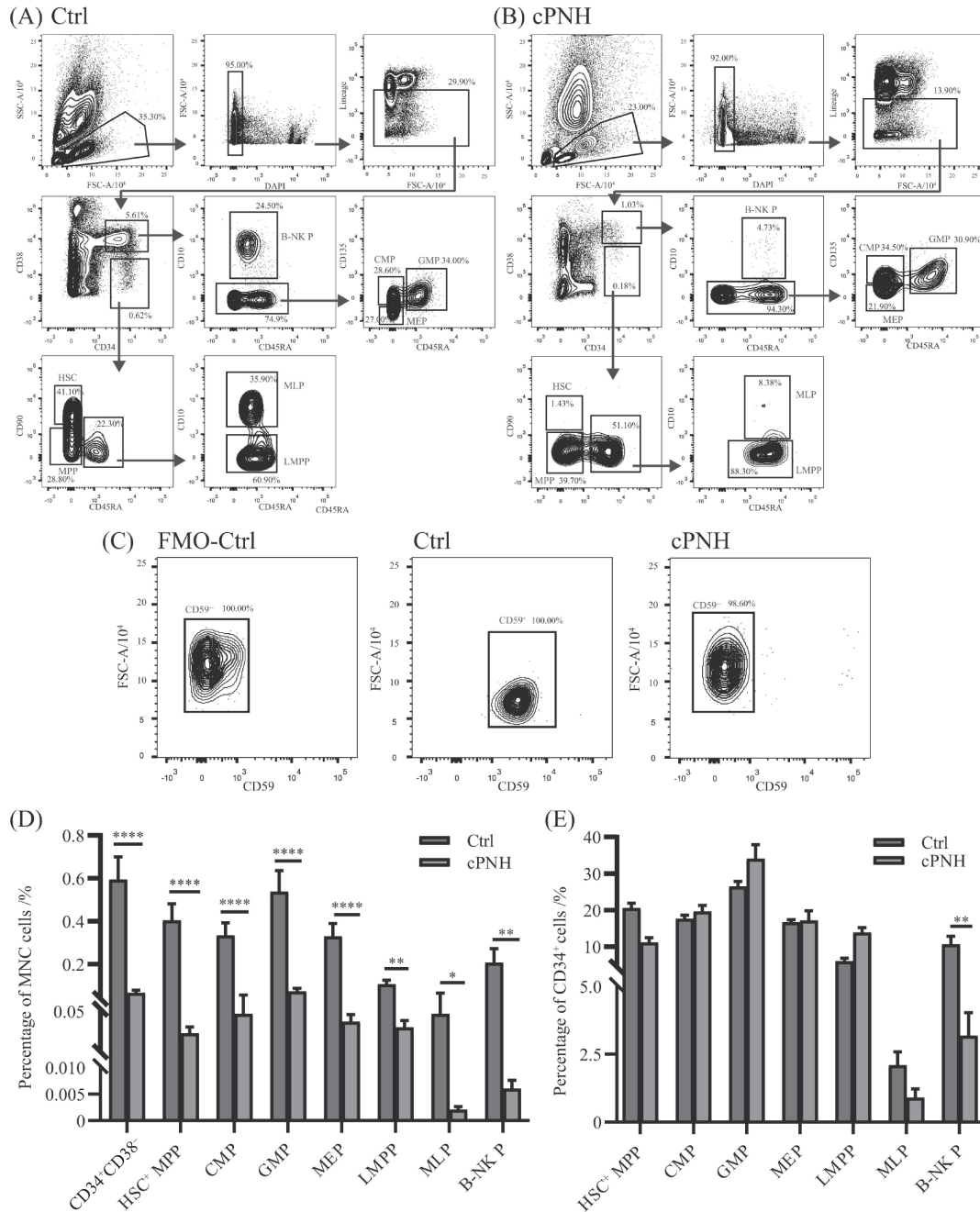
A、B: 健康对照组(Ctrl)和经典型PNH(cPNH)骨髓(BM)中分化细胞流式代表图; C: 骨髓各类血细胞比例。**P*<0.05, 经典型PNH与健康对照组相比。Ctrl: *n*=3; cPNH: *n*=4。

A,B: representative flow plots for blood cells from BM from a Ctrl and a cPNH case; C: the proportion of blood cells in BM. **P*<0.05 cPNH vs Ctrl group. Ctrl: *n*=3; cPNH: *n*=4.

图3 骨髓中各血细胞分类及PNH克隆统计
Fig.3 Statistics of blood cells and PNH clones in BM

验检测(图5A), 结果显示, cPNH患者HSPCs体外集落形成能力明显高于健康对照组[图5B和图5C, cPNH(44%±2.54%) vs Ctrl(34.11%±2.59%); $P<0.05$].

其中, 两组在CFU-GM和CFU-GEMM数量无明显差异; 但患者祖细胞形成红系克隆的数量较对照组明显增多[图5C和图5D, cPNH(17.44%±2.07%)

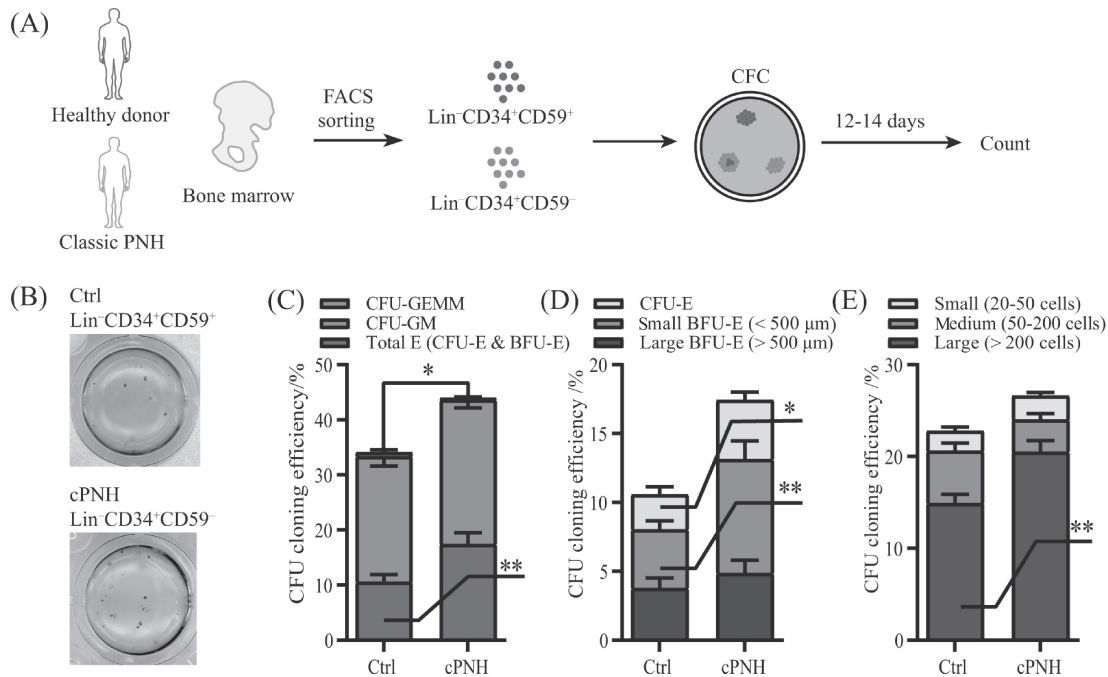


A、B: 健康对照组(Ctrl)和经典型PNH(cPNH)骨髓(BM)中HSPCs流式代表图; C: 在健康对照(左)、FMO对照(中)、经典型PNH(右)三组中, CD34⁺ HSPCs细胞PNH克隆的流式代表图, 比例为CD34⁺细胞中CD59⁺或CD59⁻细胞的内容; D: 骨髓各造血干细胞亚群占单个核细胞(MNCs)的比例; E: 骨髓各HSPCs亚群占CD34⁺HSPCs细胞的比例。* $P<0.05$, ** $P<0.01$, **** $P<0.0001$, 经典型PNH与健康对照组相比。Ctrl: $n=12$; cPNH: $n=13$ 。

A,B: representative flow plots for HSPCs from BM from a Ctrl and a cPNH case; C: representative flow plots for PNH clones in CD34⁺ HSPCs from a Ctrl (left), a FMO-Ctrl (center) and a cPNH (right) case; and the percentage of CD59⁺ or CD59⁻ population in CD34⁺ compartment is shown; D: the proportion of HSPC subgroups in MNCs from BM; E: the proportion of HSPC subgroups in CD34⁺ HSPCs from BM. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$ cPNH vs Ctrl group. Ctrl: $n=12$; cPNH: $n=13$.

图4 骨髓中各干祖细胞亚群及CD34⁺细胞PNH克隆统计

Fig.4 Statistics of HSPCs and PNH clones in CD34⁺ HSPCs in BM



A: 实验流程图; B: CFU代表图; C: Ctrl组HSPCs(Lin⁻CD34⁺CD59⁺)和cPNH组HSPCs(Lin⁻CD34⁺CD59⁺)体外集落形成统计; D: E系集落统计; E: GM系集落统计。* $P < 0.05$, ** $P < 0.01$, 经典型PNH与健康对照组相比。

A: experimental flow diagram; B: representative photos of CFU; C: statistics of colony forming ability of HSPCs (Lin⁻CD34⁺CD59⁺) from the Ctrl group and HSPCs (Lin⁻CD34⁺CD59⁺) from the cPNH group; D: statistics of E colonies; E: statistics of GM colonies. * $P < 0.05$, ** $P < 0.01$ cPNH vs Ctrl group.

图5 经典型PNH和对照组CD34⁺ HSPCs的CFU实验

Fig.5 CFU assays for CD34⁺ HSPCs from the cPNH and Ctrl group

vs Ctrl(10.58%±1.3%); $P < 0.01$ 。此外, 红系克隆中, 患者组形成CFU-E和small BFU-E的数量明显多于对照组[图5D, CFU-E: cPNH(4.3%±0.58%) vs Ctrl(2.53%±0.57%), $P < 0.05$; small BFU-E: cPNH(8.28±1.33%) vs Ctrl(4.27%±0.6%), $P < 0.01$]。而在粒、巨噬细胞集落形成单位中, 患者组产生了更多的Large CFU-GM克隆[图5E, cPNH(20.5%±1.2%) vs Ctrl(14.91%±0.98%), $P < 0.01$]。

3 讨论

PNH是一种罕见的获得性HSCs克隆性疾病。致使HSCs发生病变的确切原因尚不清楚。致病因素可能有化学、放射线或病毒感染等。HSCs致病染色体发生突变, 产生异常干细胞株, 其增殖、分化生成的红细胞、粒细胞和淋巴细胞等都有共同缺陷。以往的研究主要通过检测患者外周血红细胞和粒细胞的CD59和FLAER的表达情况来判断PNH克隆的大小, 对患者外周血、骨髓中各类血细胞以及HSPCs的检测尚未有报道。本研究通过流式细胞术、体外集落形成实验等方法检测和比较cPNH患者与

健康对照组外周血以及骨髓中的血细胞和造血干祖细胞的数量及功能差异, 研究PNH异常克隆生物学特征。

在外周血细胞中cPNH患者T细胞、B细胞和NK细胞数量明显减少, 髓系细胞数无明显改变。骨髓各类血细胞中, cPNH患者单核细胞比例明显高于对照组, 中性粒细胞、有核红细胞以及淋系细胞比例无明显改变。骨髓HSPCs的流式结果显示, cPNH患者HSPCs几乎全部为CD59⁺的PNH异常克隆, 其异常克隆的比例与在外周血和骨髓中成熟粒、单核细胞检测的比例一致。cPNH患者HSPCs细胞比例明显减少, 提示造血干细胞受损并大量耗竭。而在下游祖细胞中, 以淋系偏向B-NKPs和MLPs降低最为明显, 该结果与外周血和骨髓中B、NK细胞数量降低一致。这提示cPNH患者淋系祖细胞受损最为严重, 淋系细胞出现大量缺失。CMPs、GMPs和MEPs在CD34⁺的HSPCs中的比例与对照组无差异, 提示cPNH患者的HSCs向CMPs、GMPs和MEPs分化无影响; 而B-NKPs在CD34⁺的HSPCs中的比例明显下降, 说明HSCs向B-NKPs分化受阻。CFU实验结果

提示, cPNH患者HSPCs形成髓系克隆的能力与健康对照组无差异;而在红系克隆中, cPNH患者形成的偏小的红系克隆明显增多,该结果提示, cPNH患者HSPCs可以形成髓系和红系克隆,且出现红系克隆代偿性增多。

此前在*PIGA*基因突变的小鼠或恒河猴的动物模型中发现, GPI-细胞与正常细胞相比,不具有克隆扩增的优势^[28-30],且单纯的*PIGA*基因的突变并不足以导致小鼠溶血^[31]和PNH发病。而近年来的一些研究发现,基因突变累积、免疫机制、抗凋亡机制、基因的异常表达以及微环境改变均可在其中发挥重要作用^[4]。我们的研究结果提示, cPNH患者异常克隆中HSPCs大量耗竭,向B-NKPs分化受阻,骨髓HSPCs检测对解析PNH克隆特征具有重要意义。

参考文献 (References)

- [1] TAKEDA J, MIYATA T, KAWAGOE K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria [J]. *Cell*, 1993, 73(4): 703-11.
- [2] KINOSHITA T, FUJITA M. Biosynthesis of GPI-anchored proteins: special emphasis on GPI lipid remodeling [J]. *J Lipid Res*, 2016, 57(1): 6-24.
- [3] UniProt: a hub for protein information [J]. *Nucleic Acids Res*, 2015, 43(Database issue): D204-12.
- [4] HILL A, DEZERN A E, KINOSHITA T, et al. Paroxysmal nocturnal haemoglobinuria [J]. *Nat Rev Dis Primers*, 2017, 3(1): 17028.
- [5] JALBERT J J, CHAUDHARI U, ZHANG H, et al. Epidemiology of PNH and real-world treatment patterns following an incident PNH diagnosis in the US [J]. *Blood*, 2019, 134(Supplement_1): 3407.
- [6] RICHARDS S J, PAINTER D, DICKINSON A J, et al. The incidence and prevalence of patients with paroxysmal nocturnal haemoglobinuria and aplastic anaemia PNH syndrome: a retrospective analysis of the UK's population-based haematological malignancy research network 2004-2018 [J]. *Eur J Haematol*, 2021, 107(2): 211-8.
- [7] MUKHINA G L, BUCKLEY J T, BARBER J P, et al. Multilineage glycosylphosphatidylinositol anchor-deficient haematopoiesis in untreated aplastic anaemia [J]. *Br J Haematol*, 2001, 115(2): 476-82.
- [8] DEZERN A E, BOROWITZ M J. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 1: clinical utility [J]. *Cytometry B Clin Cytom*, 2018, 94(1): 16-22.
- [9] SUN L, BABUSHOK D V. Secondary myelodysplastic syndrome and leukemia in acquired aplastic anemia and paroxysmal nocturnal hemoglobinuria [J]. *Blood*, 2020, 136(1): 36-49.
- [10] HALL S, ROSSE W. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria [J]. *Blood*, 1996, 87(12): 5332-40.
- [11] SUTHERLAND D R, ILLINGWORTH A, MARINOV I, et al. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 2: reagent selection and assay optimization for high-sensitivity testing [J]. *Cytometry B Clin Cytom*, 2018, 94(1): 23-48.
- [12] SUTHERLAND D R, KUEK N, DAVIDSON J, et al. Diagnosing PNH with FLAER and multiparameter flow cytometry [J]. *Cytometry B Clin Cytom*, 2007, 72(3): 167-77.
- [13] PARKER C, OMINE M, RICHARDS S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria [J]. *Blood*, 2005, 106(12): 3699-709.
- [14] CANNIZZO E, RAI A M, DE PROPRIIS M S, et al. Features, reason for testing, and changes with time of 583 paroxysmal nocturnal hemoglobinuria clones from 529 patients: a multicenter Italian study [J]. *Ann Hematol*, 2019, 98(5): 1083-93.
- [15] RICHARDS S J, WHITBY L, CULLEN M J, et al. Development and evaluation of a stabilized whole-blood preparation as a process control material for screening of paroxysmal nocturnal hemoglobinuria by flow cytometry [J]. *Cytometry B Clin Cytom*, 2009, 76(1): 47-55.
- [16] HU R, MUKHINA G L, PIANTADOSI S, et al. PIG-A mutations in normal hematopoiesis [J]. *Blood*, 2005, 105(10): 3848-54.
- [17] BABUSHOK D V. When does a PNH clone have clinical significance [J]? *Hematology*, 2021, 2021(1): 143-52.
- [18] ARATEN D J, NAFA K, PAKDEESUWAN K, et al. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals [J]. *Proc Natl Acad Sci USA*, 1999, 96(9): 5209-14.
- [19] SHEN W, CLEMENTE M J, HOSONO N, et al. Deep sequencing reveals stepwise mutation acquisition in paroxysmal nocturnal hemoglobinuria [J]. *J Clin Invest*, 2014, 124(10): 4529-38.
- [20] FRAIMAN Y S, CUKA N, BATISTA D, et al. Development of paroxysmal nocturnal hemoglobinuria in CALR-positive myeloproliferative neoplasm [J]. *J Blood Med*, 2016, 7: 107-10.
- [21] GARGIULO L, ZAIMOKU Y, SCAPPINI B, et al. Glycosylphosphatidylinositol-specific T cells, IFN- γ -producing T cells, and pathogenesis of idiopathic aplastic anemia [J]. *Blood*, 2017, 129(3): 388-92.
- [22] KUNYABOON R, WANACHIWANAWIN W, PRATYA Y U, et al. Mechanism of paroxysmal nocturnal hemoglobinuria clonal dominance: possible roles of different apoptosis and CD8⁺ lymphocytes in the selection of paroxysmal nocturnal hemoglobinuria clones [J]. *Hematol Oncol Stem Cell Ther*, 2012, 5(3): 138-45.
- [23] CHEN G, KIRBY M, ZENG W, et al. Superior growth of glycosylphosphatidylinositol-anchored protein-deficient progenitor cells in vitro is due to the higher apoptotic rate of progenitors with normal phenotype *in vivo* [J]. *Exp Hematol*, 2002, 30(7): 774-82.
- [24] BRODSKY R A, VALA M S, BARBER J P, et al. Resistance to apoptosis caused by PIG-A gene mutations in paroxysmal nocturnal hemoglobinuria [J]. *Proc Natl Acad Sci USA*, 1997, 94(16): 8756-60.
- [25] 沈悌. 血液病诊断及疗效标准, 第4版[M]. 北京: 科学出版社, 2018.
- [26] CELL ANALYSIS COMMITTEE, CHINESE SOCIETY

- OF BIOTECHNOLOGY CLINICAL FLOW CYTOMETRY GROUP, BLOOD IMMUNE COMMITTEE CHINESE SOCIETY OF IMMUNOLOGY RED BLOOD CELL DISEASE GROUP, CHINESE SOCIETY OF HEMATOLOGY CHINESE MEDICAL ASSOCIATION. Chinese expert consensus on paroxysmal nocturnal hemoglobinuria detection via flow cytometry (2021) [J]. *Zhonghua Xue Ye Xue Za Zhi*, 2021, 42(4): 281-7.
- [27] ILLINGWORTH A, MARINOV I, SUTHERLAND D R, et al. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 3: data analysis, reporting and case studies [J]. *Cytometry B Clin Cytom*, 2018, 94(1): 49-66.
- [28] MURAKAMI Y, KINOSHITA T, MAEDA Y, et al. Different roles of glycosylphosphatidylinositol in various hematopoietic cells as revealed by a mouse model of paroxysmal nocturnal hemoglobinuria [J]. *Blood*, 1999, 94(9): 2963-70.
- [29] KELLER P, TREMML G, ROSTI V, et al. X inactivation and somatic cell selection rescue female mice carrying a Piga-null mutation [J]. *Proc Natl Acad Sci USA*, 1999, 96(13): 7479-83.
- [30] SHIN T H, BAEK E J, CORAT M A F, et al. CRISPR/Cas9 PIG: a gene editing in nonhuman primate model demonstrates no intrinsic clonal expansion of PNH HSPCs [J]. *Blood*, 2019, 133(23): 2542-5.
- [31] CHEN Y, RONG F. Advances in the creation of animal models of paroxysmal nocturnal hemoglobinuria [J]. *Hematology*, 2021, 26(1): 491-6.