



HER2基因组DNA标准物质互通性研究*

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摘要 采用实验室建立的数字PCR方法(ddPCR)和荧光定量PCR(qPCR)方法研究HER2基因组DNA标准物质的互通性, 评价HER2基因组DNA标准物质与临床样本的互通性, 为临床实验室检测提供具有互通性的可溯源标准物质. 将研制的5个水平标准物质随机穿插于29例临床样本间, 使用ddPCR方法与qPCR方法同时进行检测. 参考美国临床实验室标准化协会(CLSI)指南文件EP30和中华人民共和国卫生行业标准基质效应与互通性评估指南WS/T356-2011的推荐, 采用Deming回归法评价标准物质的互通性. 结果显示, 5种标准物质与临床样本之间具有良好的互通性, 可以用于临床实验室对HER2基因拷贝数变异检测方法的方法验证和质量控制.

关键词 乳腺癌基因检测, 数字PCR, HER2基因组DNA标准物质, 拷贝数变异

中图分类号 Q7, R4

DOI: 10.16476/j.pibb.2020.0404

乳腺癌是女性中常见的恶性肿瘤, HER2基因是临床诊断乳腺癌最常用的标志物之一^[1-3], 其表达对乳腺癌具有高度特异性, 常用于筛查原发性乳腺癌高危人群, 诊断和监测治疗结果及肿瘤复发情况, 该基因的准确测量对原发性乳腺癌的诊断和患者管理的决策具有重要意义. 现有的乳腺癌HER2检测^[4]指南推荐的方法主要为免疫组化(IHC)和荧光原位杂交法(FISH), 这些方法通常依赖于医生的经验判断, 相比而言, 数字PCR(ddPCR)和荧光定量PCR(qPCR)方法具有更多的客观优势^[5-6]. 此外, 在不同的临床实验室, 选用的测量方法和仪器也各不相同, 对测量结果的准确性也存在一定的差异, 尽管现有的标准有助于临床实验室检测结果的标准化, 但缺乏互换性的标准物质用于评价检测方法的可靠性, 所以测量方法的使用也会受到局限. 互通性, 也称互换性, 是指参考物质与具有代表性的临床样本在不同测量系统结果之间数学关系的等效性^[7-10]. 因此, 不同测量方法对HER2基因的准确分析有赖于具有互通性的标准物质.

本研究基于中国计量科学研究院(National

Institute of Metrology, NIM)参与国际比对的数字PCR平台, 研制了5个水平的HER2基因组DNA标准物质, 使用来源于乳腺癌组织样本的29份基因组DNA, 来评估标准物质与临床样本在ddPCR和qPCR平台之间的互通性, 以期为临床或实验室研究选用合适的检测方法和标准物质提供参考.

1 材料与方法

1.1 检测样本

29例浸润性乳腺癌组织样本, 由火箭军特色医学中心提供, 标本送检时已告知患者, 其送检样本除用于病理诊断外, 还可能用于非营利目的的科学研究, 所有乳腺癌患者均知情同意.

* 国家质量基础的共性技术研究与应用专项课题(2017YFF0204605)和人类生殖遗传资源服务管理云平台开发与安全标准规范体系建设(2016YFC1000301-3)资助项目.

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收稿日期: 2020-11-13, 接受日期: 2021-03-03

1.2 标准物质

取*HER2*高拷贝的HCC1954细胞基因组, 与正常人永生化细胞系基因组混合配制. 共配制5个水平, *HER2*拷贝数比值在2.0~36.0 (表1).

Table 1 <i>HER2</i> reference material information				
Reference materials	<i>HER2</i>	<i>RPPH1</i>	<i>HER2/RPPH1</i>	
	/(cp·μl ⁻¹)	/(cp·μl ⁻¹)	Ratio	RSD
NIM- <i>HER2</i> -A	57 281.84	30 086.21	1.91	2.49%
NIM- <i>HER2</i> -B	172 193.38	30 079.81	5.70	3.74%
NIM- <i>HER2</i> -C	566 204.87	33 469.56	16.94	4.01%
NIM- <i>HER2</i> -D	808 548.81	36 137.10	22.38	4.02%
NIM- <i>HER2</i> -E	1417 869.31	40 179.04	35.38	7.67%

1.3 仪器与试剂

QX200微滴生成仪、QX200微滴检测仪及其控制分析软件均购自伯乐生命医学产品(上海)有限公司, 相关的消耗品包括微滴发生卡(批号: 1864008)、96孔板(批号: 0030128605)、密封膜(批号: 1814040), 罗氏荧光定量PCR仪(型号: Lightcycler 480). 基因组提取试剂盒为QIAGEN Blood & Cell Culture DNA Maxi Kit 500G(批号: 13362). *HER2*基因及内参基因引物探针由华大基因合成.

1.4 *HER2*基因ddPCR定量方法

使用*HER2*基因拷贝数浓度均值与内参基因(*RPPH1*)拷贝数浓度的比值, 作为*HER2*基因拷贝数比值定量结果. 中国计量院在DNA拷贝数测量能力方面通过国际比对验证^[11], 获得了国际互认的校准测量能力^[12], 因此定量结果可溯源至该项测量能力. *HER2*基因引物探针浓度为300 nmol/L, *RPPH1*基因引物浓度为250 nmol/L, 探针浓度为100 nmol/L, 退火温度60℃^[13].

1.5 统计分析

使用Microsoft Excel 2016处理数据, 结果依据美国临床和实验室标准协会(CLSI)指南文件EP14-A3^[14] (Clinical and Laboratory Standards Institute. Evaluation of commutability of processed samples; Approved guideline-Third edition: EP14-A3)和中华人民共和国卫生行业标准基质效应与互通性评估指南(WS/T 356-2011)关于互通性评价推荐的指南方法^[15], 使用Deming回归法评估实验室自制*HER2*标准物质在ddPCR与qPCR平台的

互通性.

1.6 评价标准

依据EP14-A3, 将两个平台的结果进行Deming回归, 以*HER2*与*RPPH1*的比值作为结果, 以ddPCR样本检测结果的均值作为横坐标, qPCR样本检测结果的均值作为纵坐标, 绘制回归曲线, 根据ddPCR临床样本检测结果, 计算相对应的*Y*值预测值与双侧95%的预测区间, 并绘制Deming回归图. 若自制的标准物质检测结果落于该区间内, 则认为其具有互通性, 若落于该区间外, 则认为其不具有互通性.

95% 预测区间=

$$\bar{y}_{Pred} \pm t(0.975, n-2) S_{y,x} \sqrt{1 + \frac{1}{n} + \frac{(\bar{x}_i - \bar{\bar{x}})^2}{\sum (\bar{x}_i - \bar{\bar{x}})^2}}$$
$$S_{y,x} = \sqrt{\frac{\sum (y_{pred} - \bar{y}_i)^2}{n - g}}$$

其中 $\bar{y}_{Pred}$ 为根据回归曲线计算出在ddPCR结果时qPCR的值; n 为样本数, 根据EP14-A3指导意见, 20例样本足以在互通性评估的研究中适用, 且中华人民共和国卫生行业标准基质效应与互通性评估指南(WS/T 356-2011)中也使用20例样本作为指南, 故本研究取用29例样本, 其 $t_{(0.975\ 27)}$ 查表为2.052; g 为常数项, 在线性回归时为2; $\bar{x}_i$ 为ddPCR第*i*个样本测定的均值; $\bar{y}_i$ 为qPCR第*i*个样本测定的均值; $\bar{\bar{x}}$ 为所有样本ddPCR法测定均值的整体均值.

1.7 FISH检测

将*HER2*高拷贝的HCC1954细胞系, 与正常人永生化细胞进行检测, 双人计数20个细胞.

2 结 果

2.1 互通性结果

2.1.1 *HER2/RPPH1*结果

通过2种方法对*HER2*基因组DNA标准物质的测量值进行比较来评估互通性, 图1展示了5种标准物质与所有临床样本使用两种方法测定的结果. Deming回归法对5种标准物质互通性的评价结果显示, 实验室*HER2*基因组DNA标准物质与临床样本在ddPCR平台与qPCR平台均具有良好的互通性, 无基质效应(表2).

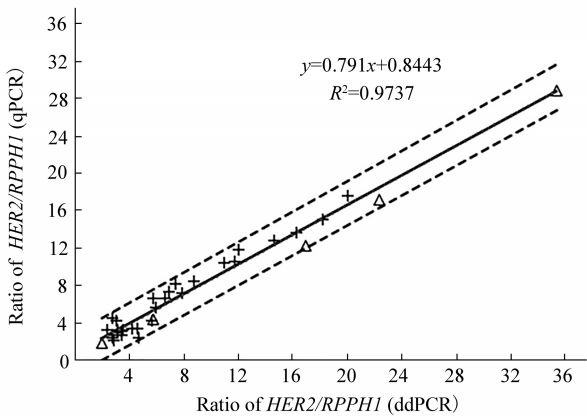


Fig. 1 Evaluation results of commutability of reference materials by Deming regression method

The ratio of *HER2/RPPH1* was used. —: Regression curve of clinical sample determination; -----: 95% confidence interval of its predicted value *y*; Δ : *HER2* reference material; +: Clinical sample. The matrix effect is judged to exist for any “ Δ ” outside the dashed line.

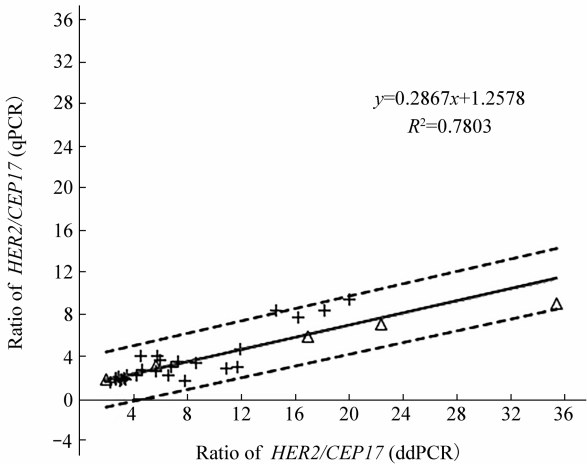


Fig. 2 Evaluation results of commutability of reference materials by Deming regression method

The ratio of *HER2/CEP17* was used. —: Regression curve of clinical sample determination; -----: 95% confidence interval of its predicted value *y*; Δ : *HER2* reference material; +: Clinical sample. The matrix effect is judged to exist for any “ Δ ” outside the dashed line.

Table 2 Matrix effect evaluation of reference materials						
Reference materials	$\bar{x}_i$	$\bar{y}_i$	$\bar{y}_{Pred}$	95% $\uparrow$	95% $\downarrow$	Matrix effect
NIM-HER2-A	1.91	1.86	2.27	4.53	0.02	No
NIM-HER2-B	5.70	4.45	5.32	7.55	3.09	No
NIM-HER2-C	16.94	12.21	14.35	16.62	12.07	No
NIM-HER2-D	22.38	17.19	18.72	21.06	16.38	No
NIM-HER2-E	35.38	28.84	29.16	31.79	26.53	No

2.1.2 HER2/CEP17结果

采用*HER2/CEP17*方法所得互通性结果如图2所示.

2.2 FISH结果

HCC1954 细胞核轮廓清晰（图3，图5），*HER2* 基因和*CEP17*分别对应红、绿荧光信号，信号点清晰可见，其中红色信号点呈簇状扩增，显示*HER2* 基因为扩增阳性结果. 双人计数20个细胞，其中*CEP17*/细胞数为6.7. 正常人永生化细胞系细胞核轮廓清晰（图4，图6），*HER2* 基因和*CEP17* 分别对应红、绿荧光信号，信号点清晰可见. 双人计数20个细胞，其中*HER2/CEP17*为1.22，*HER2*/细胞数为2.6，*CEP17*/细胞数为2.13，结果为*HER2* 基因扩增阴性.

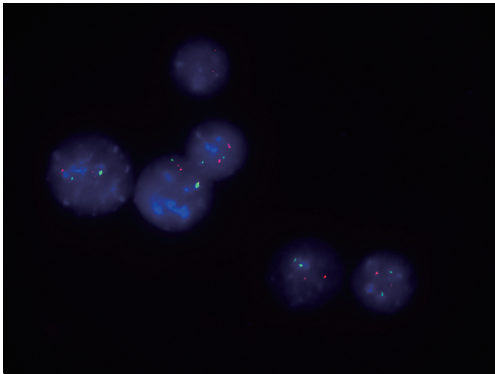


Fig. 3 FISH results of HCC1954 cells

Nucleus: DAPI staining (blue fluorescence); *HER2* gene: red fluorescence signal; *CEP17*: green fluorescence signal; *CEP17*/cell number is 6.7.

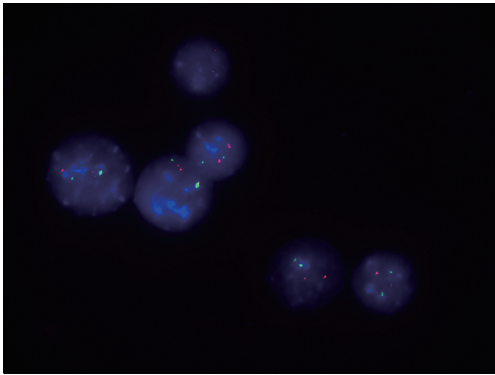


Fig. 4 FISH results of PLCL7 cells

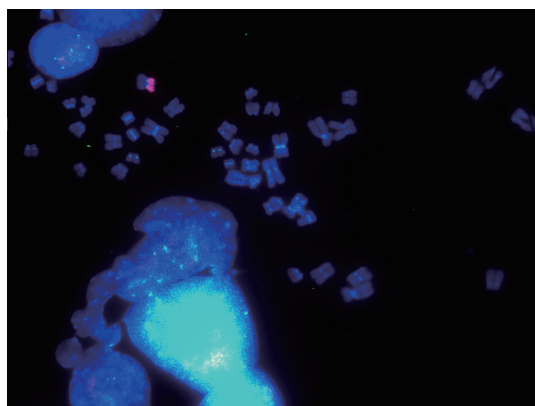


Fig. 5 Enlarged image of FISH results of HCC1954 cells in metaphase

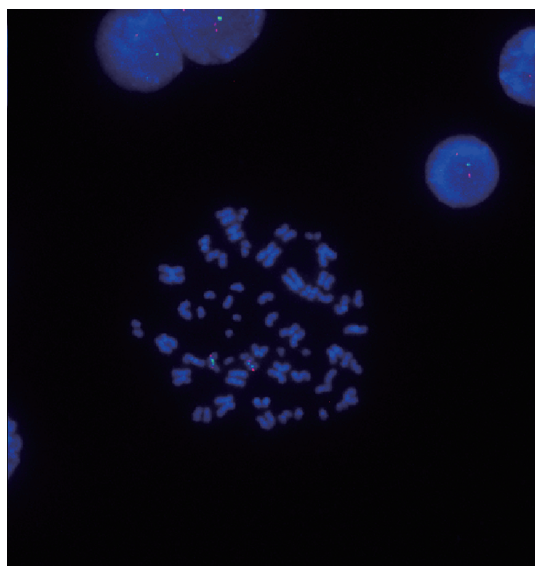


Fig. 6 Enlarged image of FISH results of PLCL7 cells in metaphase

4 讨 论

我国乳腺癌发病率居女性恶性肿瘤首位,有研究^[3]发现大多数乳腺癌患者的*HER2*基因突变导致了肿瘤的发生,*HER2*阳性乳腺癌约占侵袭性乳腺癌的20%~30%,所以对*HER2*基因的准确监控,有助于早期诊断、精准治疗及预后评估^[5].*HER2*基因是原癌基因,列属于表皮生长因子受体(epidermal growth factor receptor, EGFR)家族,定位于17号染色体长臂端,在表皮细胞的生长分化过程中具有重要的调控作用^[16].现有对*HER2*蛋白表达水平的推荐检测方法一般采用IHC法,对该基因扩增水平的推荐检测一般为原位杂交法(包括

FISH、CISH和SISH),一般临床检测上将两种方法结合使用(IHC+FISH).对IHC法结果为3+的样本判断为*HER2*阳性,对IHC 0或1+的结果判断为*HER2*阴性,对IHC 2+的结果再进行原位杂交检测(一般采用FISH法),探针多选用含有*HER2*基因和该基因所在的17号染色体着丝粒(*CEP17*)序列的双探针,在处理好的FISH切片上选择两个以上的 ≥ 20 个浸润性癌细胞的具有代表性区域,由两名以上的专业医生或操作人员对*HER2*与*CEP17*信号进行计数及比值计算,将所得结果分为*HER2/CEP17* ≥ 2.0 和 < 2.0 两种情况,在每种情况下又细分2~3种条件^[4].对结果的详细归类与解释虽然增加了结果的判读严谨性,但由于这些要建立在医师和操作人员主观观察记录的条件下,且FISH检测的技术操作严苛^[17],对环境及设备要求较高,所以在一定程度上降低了结果的可信程度.在临床上,由于尚缺少有证标准物质作为溯源标准,故而检测结果常作为参考.而通过ddPCR和qPCR方法所得到的结果依赖于客观的拷贝数值,摆脱了主观因素的影响,所以近年来在实验室的检测上常被使用.相比于qPCR,ddPCR在实验室之间和实验室内部的检测技术差异性具有明显优势^[18-19],其双重性与定量的结果使其准确度要优于qPCR,这更便于实验室之间标准的建立与优化.

本研究基于经国际互认的数字PCR测量能力^[11],研制*HER2*基因组DNA标准物质,力求为我国临床*HER2*分子检测提供可溯源的参考标准.选择*HER2*的7号外显子引物探针和作为内参的*RPPH1*基因,采用其比值作为最终的标准值,参考美国临床肿瘤学学会关于*HER2*检测指南^[20]的建议,将比值 > 2.0 的结果看作阳性.计算所有29例临床阳性样本分别在ddPCR平台和qPCR平台的*HER2*与*RPPH1*比值结果,依据美国临床实验室标准化协会(CLSI)指南文件EP30和中华人民共和国卫生行业标准基质效应与互通性评估指南WS/T356-2011的建议,将绝对定量结果更准确可信的ddPCR结果作为X轴,qPCR结果作为Y轴,绘制散点图并做Deming回归,根据每个临床样本的ddPCR平台测定值计算y值的95%置信区间,在结果中qPCR平台测定的标准物质值落在该区间内,说明该标准物质对qPCR平台无基质效应,即5种*HER2*基因组DNA标准物质与临床样本在ddPCR平台和qPCR平台间具有互通性,可以应用于临床实验室对*HER2*基因拷贝数变异检测方法的方法验

证和质量控制。

此外,由于*CEP17*基因常作为FISH检测的参考基因,所以我们同时对比了采用*HER2/CEP17*方法检测的结果(图2)。Deming回归结果显示,采用*CEP17*作为内参时,标准物质在两种方法间依旧具有良好的互通性,但所得结果的回归系数($R^2=0.78$)远小于采用*RPPH1*作为内参的所得结果($R^2=0.97$),且有一组临床样本落于95%区间外。对于这一现象,我们将两种平台测定的临床样本结果与FISH结果进行了比对,同时将用于制备*HER2*基因组DNA标准物质的细胞系进行了FISH检测(图3、4),将结果与ddPCR方法结果进行对比。结果显示,在个别细胞中存在FISH结果与ddPCR结果差值较大的情况。在HCC1954细胞FISH结果中(图5),我们发现在同一细胞核内,部分17号染色体呈现*HER2*基因于短臂扩增阳性,部分17号染色体呈现*HER2*基因扩增阴性,且*CEP17*基因存在扩增现象,而在PLCL7细胞中无此现象(图6),因此在使用*CEP17*基因作为内参基因时,可能会造成假阴性,该结论证实了本研究采用*RPPH1*基因作为内参的必要性。

4 结 论

本研究对比了研制的5种*HER2*基因组DNA标准物质与临床样本在ddPCR平台和qPCR平台之间的互通性,结果显示该5种标准物质与临床样本在两种平台间具有良好的互通性,ddPCR拷贝数比值定量方法优于传统的检测方法。此外,对比了*HER2/RPPH1*定量方法与*HER2/CEP17*定量方法之间的差别,并经FISH检测验证了*HER2*基因会存在局部扩增的情况,不建议使用*CEP17*基因作为内参基因。

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Commutability of *HER2* Genomic DNA Reference Materials*

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Abstract To evaluate the commutability between *HER2* genomic DNA reference materials (RM) and clinical samples, ddPCR and qPCR were used to study the commutability of *HER2* genomic DNA RM, which can provide traceable RM with commutability for clinical laboratory testing. 29 clinical samples were randomly measured among the 5 levels of RM by real time quantitative PCR (qPCR) and droplet digital PCR (ddPCR). The averaged copy number ratio of *HER2* to *RPPH1* was calculated. According to the guideline EP30 of American Society for clinical laboratory standardization (CLSI) and the guideline WS/t356-2011 for *Guideline for Evaluation of Matrix Effects and Commutability* of the People's Republic of China, a regression curve was drawn with the result of ddPCR as abscissa and the result of qPCR as ordinate, and the commutability of RM was evaluated by Deming regression method. If the test results of the proposed RM fall within the 95% confidence interval of its predicted, it is considered that the analyzed RM is commutable; if it falls outside the range, it is considered that the analyzed RM is not commutable. In addition, the results were compared with the ratio of copy number concentration when *CEP17* gene was used as reference gene. *HER2/RPPH1* of the five RMs determined by ddPCR and qPCR were: 1.91, 1.86; 5.70, 4.45; 16.94, 12.21; 22.38, 17.19; 35.38, 28.84, respectively, which fall in the prediction range, indicating the five RMs are commutable. Moreover, this was confirmed by the ratio of *HER2/CEP17* determined by the two methods. However, the regression coefficient of *HER2/RPPH1* ($R^2 = 0.97$) was better than that of *HER2/CEP17* ($R^2 = 0.78$) and even one group of clinical sample fell outside the 95% confident interval. We used FISH to detect the cell lines used to prepare *HER2* genomic DNA RM. The results showed that in the same nucleus, part of chromosome 17 showed *HER2* amplification in the short arm, and part of chromosome 17 showed no *HER2* amplification. Additionally, *CEP17* was amplified in some cells, which indicates it is not suitable to be used as reference gene as this will cause false negative results. This confirms the necessity of using *RPPH1* gene as reference gene when diagnostic of *HER2*. In conclusion, the five RMs are commutable, which can be used for the method validation and quality control in analyzing of *HER2* copy number variation in clinical laboratories.

Key words breast cancer gene detection, digital PCR, *HER2* genomic DNA reference material, copy number variation

DOI: 10.16476/j.pibb.2020.0404

* This work was supported by grants from Research and Application of Common Technology of National Quality Foundation (2017YFF0204605) and Development of Human Reproductive Genetic Resources Service Management Cloud Platform and Construction of Safety Standard System (2016YFC1000301-3).

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Received: November 13, 2020 Accepted: March 3, 2021