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·基础研究·

同源肿瘤克隆细胞蛋白表达和侵袭能力的异质性研究*

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摘要 目的：比较同源肿瘤细胞来源的不同单克隆表型差异。**方法：**采用极限稀释法，在悬浮培养条件下获取 HCT116 结肠癌细胞系的单个细胞，对每孔含单个的细胞进行扩增培养，获得子代单克隆，并以同样方法继续挑取单克隆，连续获得子三代克隆。根据单克隆形态特点，选取第三代的三株代表性的单克隆，采用 Western blot 和免疫荧光法比较其 SOX2、EpCAM 和 Vimentin 蛋白表达差异。采用放疗观察三株单克隆的 Vimentin 蛋白的动态变化，研究其放疗干预的时间异质性，Transwell 体外侵袭实验比较克隆侵袭力的差异。**结果：**三株由单细胞扩增培养的同源第三代子克隆依然存在明显生物学差异。形态有明显区别的球形与不规则的克隆形态。不规则形态克隆更表现为 SOX2 低表达及 Vimentin 的高表达。并且在单个细胞水平上，同个单克隆群体内也存在个体细胞间蛋白的表达差异（Vimentin; EpCAM）。通过观察放疗前后 Vimentin 蛋白在不同时间点上的荧光强度，发现肿瘤单克隆细胞存在时间异质性。Transwell 体外侵袭实验也显示三个同源克隆间存在明显的差异性。**结论：**同源的、连续单细胞扩增获得的第三代单克隆依然存在明显生物学差异，提示肿瘤内部异质性是其固有特征，并且在治疗干预下，也会引起肿瘤时间异质性的产生。

关键词：肿瘤异质性；单克隆；同源

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Research on Heterogeneity of Protein Expression and Invasion among Homologous Tumor Clones*

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ABSTRACT Objective: To investigate the phenotypic differences among homologous tumor clones derived from a single cancer cell. **Methods:** Using limited dilution method, the HCT116 colon cancer cells from the suspended culture condition were dissociated into single-cell suspension and seeded in ultra low attachment plates with average one cell per well. Within several weeks, the progeny clones derived from a single cell were obtained and expanded. By the same limited dilution method, the second and third generations of sub-monoclones, derived from one of their ex-generation clones respectively, were also acquired and expanded. Then, we chose the three representative clones of the third generation based on their distinct morphology for the further experiment. The expression of SOX2, EpCAM and Vimentin were detected via western blot or immunofluorescence. Through X-ray irradiation, the temporal heterogeneity of the three representative clones was evaluated by their dynamic alteration of Vimentin expression before and after treatment. The invasive ability of the three clones was also compared by transwell assay. **Results:** The considerable diversity of biological phenotype (eg: morphology; protein expression; invasive ability) was still present among the three clones, even they were the homologous progeny of third generation derived from a single HCT116 cancer cell. Their morphology was obvious differences (irregular or well-rounded shape). The protein expression of the irregular clone (expression of SOX2low and Vimentin high) was also different from other clones. Furthermore, the distinct protein expression was present at the single-cellular level (Vimentin; EpCAM), even they were among the same clone expanded from a single cancer cell. When the three homologous clones were treated with radiation, the temporal heterogeneity of Vimentin protein expression was present. The distinct invasive ability was also observed among the three clones. **Conclusions:** There was still obvious biological heterogeneity among the third-generation clones, even they derived from consecutive single-cell expansion. It provided the evidence to consider that the heterogeneity is the tumor inherent characteristics, and the influence of treatment intervention will also induce the temporal heterogeneity in the tumor cells.

Key words: Tumor heterogeneity; Monoclonal; Homologous

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前言

现代医学认为恶性肿瘤多是单克隆起源的[1-4]。但研究也表明,多数肿瘤存在广泛瘤内异质性。即使标本取自同组织来源的肿瘤细胞群体也可按其形态、基因组和生物学特征分为不同亚类。肿瘤从单个或少数的初始细胞发展至整体异质性的过程一直存在诸多不确定性。可能涉及的方面较广,如体内微环境、治疗干预[5]以及肿瘤基因不稳定、错配修复能力异常等[6-9]。

随着研究技术的不断更新,许多肿瘤检测已达到了单细胞水平。尤其近几年循环肿瘤细胞技术的发展,使临床医师能无创、多次、实时地监测肿瘤疾病的演变过程成为了可能。根据富集外周细胞的特殊蛋白表达(如:CD45、CK、EpCAM、Vimentin),能有效区分正常血源性细胞和异常潜在引起转移和复发的肿瘤细胞[10,11]。并且许多临床研究已显示,表皮-间质化转变相关蛋白(EpCAM、Vimentin)的表达高低与肿瘤疾病的预后密切相关[10]。不过,随着进一步的研究表明,不管是瘤内细胞还是游离的循环肿瘤细胞,其相关生物学特性依然存在相当大的异质性,同一病人来源的循环肿瘤细胞也可根据相应特征分成多个亚类细胞群体,肿瘤细胞在不同疾病时相和状态下处于动态的转变过程[11]。

因此,为更好理解肿瘤异质性的根本特性,本研究通过对具有相同遗传背景的同源肿瘤子克隆株进行蛋白表达和侵袭能力差异作比较,以探讨肿瘤细胞本身内部因素所能产生异质性的程度,并且运用放疗手段,观察由单一外部因素干预,造成肿瘤相关表-间质化蛋白表达(Vimentin)的时间异质性。

1 材料与方法

1.1 材料

1.1.1 细胞与试剂 HCT 116,人结肠癌细胞,购自中国科学院上海生命科学研究院细胞库。

DMEM 高糖培养基购自美国 Gibco 公司。Transwell 小室购自 Millipore 公司。各类抗体试剂购自美国 Cell Signaling Technology 公司。免疫荧光试剂购自美国 Cytelligen 公司。

1.1.2 主要仪器 超净工作台购自美国 Thermo Fisher 公司。生物安全柜购自美国 Nuaire 公司。二氧化碳恒温培养箱购自美国 Thermo Fisher 公司。各种量程微量移液器购自德国 Eppendorf 公司。细胞培养皿、各种类型孔板、离心管以及冻存管购自美国 Corning 公司。

1.2 方法

1.2.1 细胞培养 将 HCT116 细胞转至超低吸附培养板或皿内,使其处于悬浮生长状态。随培养时间延长,逐渐筛选出适应悬浮条件下的细胞团。正常消化离心和传代。培养条件为含有 10 %血清的 DMEM 高糖培养基。37 ℃,5 % CO_2 恒温培养箱。

1.2.2 单克隆扩增 预先计数每 mL 所含有的细胞数量,然后利用极限稀释法(梯度浓度对倍稀释法),将悬浮状态下的细胞最终以各排培养孔中按每孔 1 个细胞的浓度接种于 96 孔板中[12]。3-5 小时后使细胞沉淀,在倒置相差显微镜下观察,严格确认,给仅有捕获单个细胞的孔标注记号,继续随后的单细胞扩增培养,获得单克隆。

1.2.3 单克隆扩增的连续子三代克隆细胞获得 运用上述同样方法,对其中一个生长良好的单克隆再次挑取单个细胞进行扩增培养,连续同样多次操作后获得第三代单克隆株。

1.2.4 Western blot 制备的凝胶按厂商指导制备相应的 SDS-聚丙烯酰胺凝胶,每孔 25 μg 上样量,然后进行后续的电泳、转模、封闭,取出相应的一抗 4 ℃过夜,二抗室温孵育 2 h,ECL 发光显色。

1.2.5 荧光染色 根据产品商说明书进行实验(美国 Cytelligen)。取悬浮细胞液,加入清洗缓冲液,洗涤数次后离心弃上清至 100 μL。加入 2 μL抗原修复破膜处理 10 分钟, 随后加入 200 μL抗原稀释液与各 1 μL荧光染料结合单克隆抗 - EpCAM、Vimentin(cytelligen,美国)加入样本中,混匀静止 20 分钟。洗涤后离心 5 分钟,弃上清至 200 μL,重复 1 次。留 100 μL 细胞沉淀并与 100 μL细胞固定剂充分混合, 均匀涂抹于载玻片上,放置 37 ℃干燥过夜。次日原位杂交(染色体探针 Cep 8 着丝粒)后,使用抗体清洗液洗涤 2 次,吸干玻片上液体,加入 DAPI 试剂 10 μL。盖上盖玻片,立即上机检测。

1.2.6 放疗射线照射 将预照射的细胞放置在设备配套的有机透明玻璃板上调整照射视野,在 ONCOR 线性加速器进行单次 10Gy 剂量的照射,剂量率为 3.6 Gy/min。

1.2.7 Transwell 体外侵袭实验 将孔径为 8 μm 的 Transwell 上室放置 24 孔板内,按 1:5 稀释的 DMEM 与基质 Matrigel 混合,基底膜 100 μL平铺上室,紫外消毒过夜后,用无血清细胞培养液轻洗凝胶。将细胞浓度调至 5× 10^5 个 /mL,1 %血清,加入上室 200 μL细胞悬液。下室加入 600 μL含有 20 %血清的 DMEM 培养液。常规培养 36 h 后,取出,然后用棉签拭去上室残留的细胞。甲醛固定后用结晶紫染色 20 min,清洗后倒扣沥干,选取各样本的镜下固定视野进行计数和统计。

1.3 统计学分析

采用 GraphPad Prism 5 软件进行数据作图和相关统计分析。实验重复三次,结果以平均值± 标准差表示。两组间数据比较用 t 检验分析,以 $*P<0.05$;$**P<0.01$;$***P<0.001$ 为显著性统计意义分类。

2 结果

2.1 子三代克隆的整体适应性和克隆形态差异

我们通过连续对 HCT116 细胞系的单个细胞扩增培养,逐渐获得了连续三代的由单个细胞衍生来源的同源克隆株。经过持续的悬浮培养环境,子三代单克隆株整体上呈现出逐渐的适应性。比较各代生长情况能发现,由单个细胞所能扩增增殖的能力越来越强(图 1A)。不过,虽然是同源的克隆,但其形态上却能分出明显不同的两类,并且并不因连续代数的单克隆操作而消失。如第三代单克隆株中,代表性的克隆 1 中,细胞群体消化传代后,在悬浮条件下培养数天后呈现出克隆细胞多为不规则形,而单克隆 2 和单克隆 3 内则更具有规则的圆形特征(图1B)。

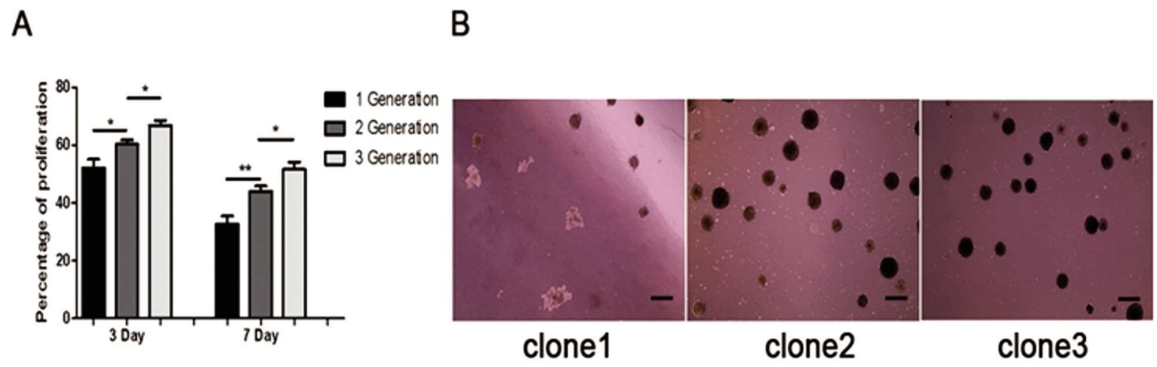

图 1 A.在第 3 和第 7 天观察的不同代数中单个细胞存活并能增殖细胞的百分比

B.代表性的第三代单克隆(克隆 1、克隆 2、克隆 3)形态差异(放大倍数:× 40)

Fig.1 A. The ratio of single cell which can viable and proliferate in different generations on day3 and day 7

B. Different morphology of represented third-generation clone (clone1; clone2; clone3; Magnification: × 40)

2.2 代表性的第三代子克隆的蛋白表达差异性

Western blot 结果显示，三株子克隆具有明显的蛋白表达差异。形态为不规则形的单克隆 1,表现为干性转录因子 SOX2 的低表达以及表皮 - 间质化标记 Vimentin 蛋白的高表达。而具有圆形形态的单克隆 2 和单克隆 3 则具有不同程度的 SOX2 的高表达以及 Vimentin 蛋白的极低表达(图 2)。

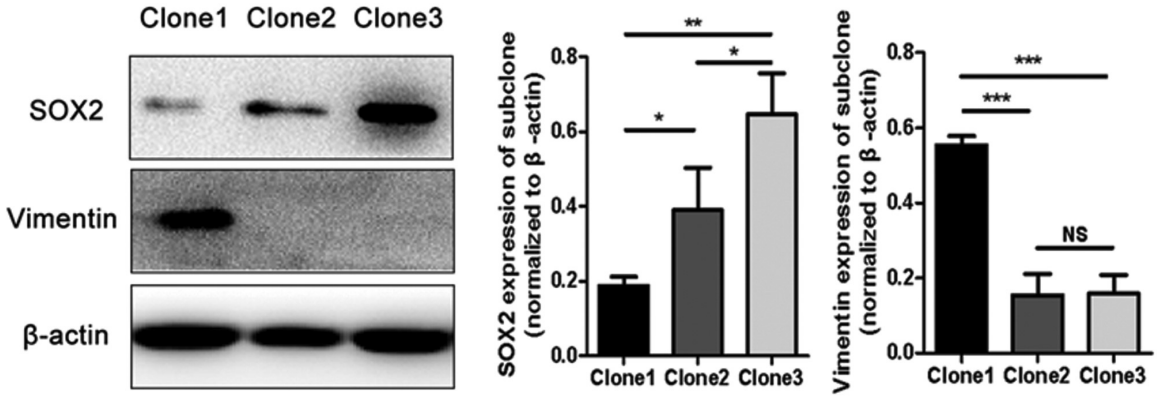

图 2 代表性的三个克隆蛋白表达差异

Fig.2 Diverse expression of protein among three clones

2.3 单细胞扩增来源的子克隆内部细胞间的差异

免疫荧光实验的结果显示,三个第三代单克隆互相之间存在 Vimentin 荧光表达的差异性。单克隆 1 的内部细胞表现出更多的 Vimentin 阳性细胞，而单克隆 2 和单克隆 3 则较少细胞表达 Vimentin。不过免疫荧光实验也同时能更清楚地观察到每个克隆内部单个细胞的水平。我们发现同个克隆细胞群体虽然是由单个肿瘤细胞分裂衍生而来的，但依然存在细胞间 Vimentin 表达的不同。单克隆 1 虽然观察到多数表达 Vimentin 的细胞,但也有个别细胞不表达;反之亦然的情况也存在于单克隆 2 和单克隆 3 中，能观察到多数细胞不表达 Vimentin,但个别细胞却表达阳性(图 3;白色箭头)。并且,同样观察 EpCAM 在克隆内部的表达情况，也能显示单个细胞层面的异质性表达(图 3;绿色箭头)。

2.4 子克隆 Vimentin 表达的时间异质性

我们对以上这三个蛋白表达差异的单克隆进行单次 10 Gy 剂量的放射处理。通过对处理前后的时间节点上 Vimentin 阳性细胞的统计，我们发现三个子克隆的 Vimentin 表达也具有治疗干预下的时间异质性。虽然在基础表达水平,这三个单克隆互相间的 Vimentin 阳性细胞比率并不同，但是经过放射后,都具有明显的规律性升降变化,表现为接受初始照射后短周期内 Vimentin 表达降低,随后在第 16 天开始又有明显的表达上升峰值出现,而后 31 天又回复之前状态(图 4)。

2.5 三个子克隆的侵袭能力差异

Transwell 体外侵袭实验结果显示，虽然是同源连续扩增的子代单克隆,但其侵袭力差异明显(图 5)。细胞能够通过上室的基质胶进入下室,在结晶紫染色后能显示大片紫色为通过孔径后的迁移细胞。具有不规则形态并且 Vimentin 高表达的单克隆 1 具有相当明显的侵袭能力。不过单克隆 2 虽然低表达 Vimentin,但也能表现出高侵袭能力。而相对于单克隆 3 则明显最弱。

3 讨论

尽管现代医学对肿瘤治疗的观念与技术水平已有了质的飞跃,也已有很多学者提出了肿瘤可作为慢性疾病的治疗策略[13],甚至有些肿瘤的确已达到可治愈的标准[14,15]。但目前多数恶性肿瘤依然在防复发、完全治愈上存在进展困难。肿瘤的异质性是其难治性的根本原因，影响治疗过程中的各种不确定性,导致同类肿瘤疾病过程中完全不同的治疗反应、预后转归[16-18]。

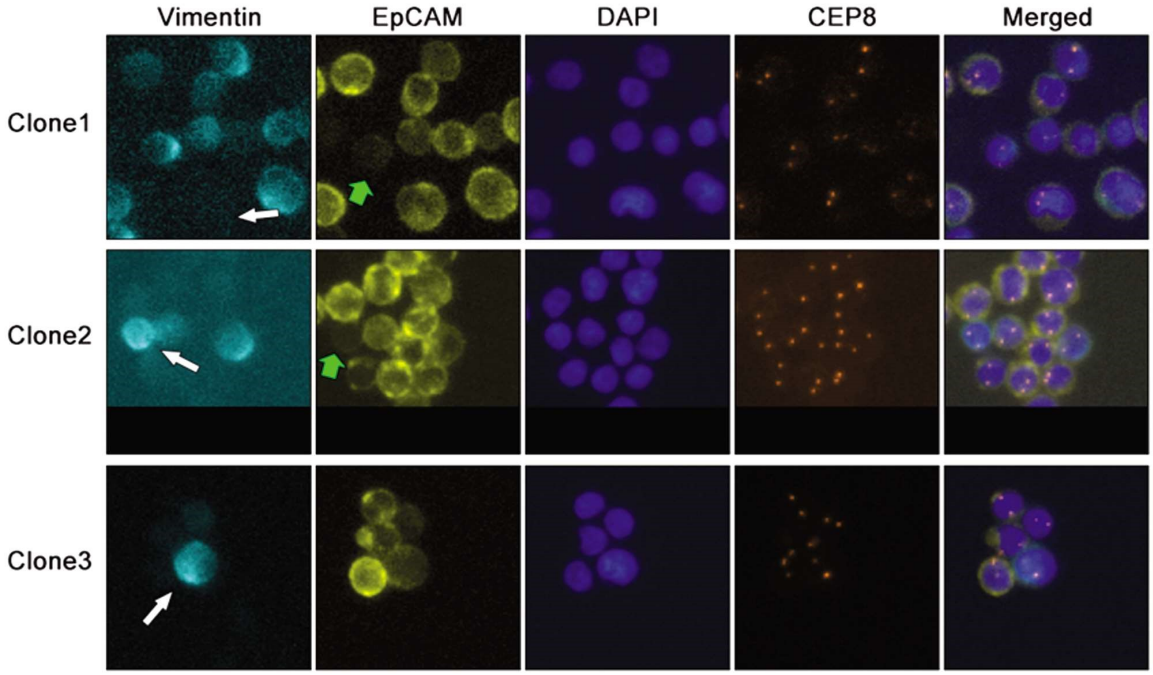

图 3 在克隆内部单个细胞水平的蛋白表达差异

Fig.3 Diverse expression of protein of single-cellular level in individual clone

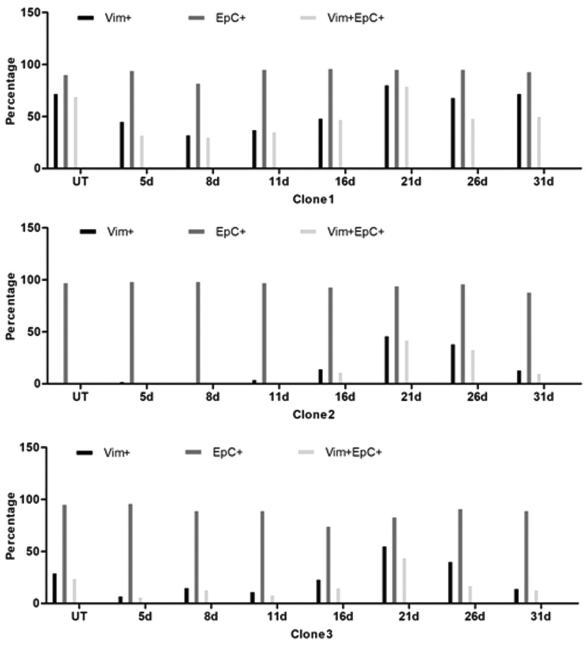

图 4 放疗前后克隆表达 Vimentin 的时间异质性

Fig.4 The temporal heterogeneity of Vimentin expression among clones before and after irradiation

国内外许多源于临床样本的研究证实，肿瘤异质性在实体瘤和血液肿瘤中广泛存在并处于动态变化的状态[19,20]。近年来已有许多文献报道运用同样的单个细胞的水平辨别不同肿瘤亚群细胞的异质性[21]。但这些相关的研究并不能完善地具体演示肿瘤异质性是如何从最初的单个恶性细胞转变成许多差异性巨大的细胞克隆群体的。在由单个肿瘤细胞这样恶性增殖的过程中，到底外因或内因各自会造成后代肿瘤细胞多大的影响并未被量化和认定。本研究通过单个肿瘤细胞不断地多次单克隆纯培养，获得了遗传背景相同的第三代同源肿瘤细胞群体。在这样的基准下，我们更好地揭示出了肿瘤内在固有的因素依然会造成其后代克隆细胞产生明显的异质性，这可解释临床许多肿瘤疾病在未受外部干预的情况下会快速发生变异。虽然我们所获得的许多同源子克隆中，的确有许多具有相似的表型特征，不过这其中也依然存在一些生物学性状差异巨大的单克隆株。

我们所选取的具有代表性的三株克隆的相关实验表明差异明显的同源肿瘤克隆株间会存在某些重要蛋白表达的异质性。SOX2 往往认为与细胞干性[22]、转录调控等相关[23]；Vimentin 的表达更认为与循环肿瘤细胞的表皮 - 间质化转变[24]、远端转移相关[11,25,26]。因此，我们在实验中也对这两类蛋白进行检测与比较。结果显示同源的这三株肿瘤克隆细胞在整体水平上 SOX2 和 Vimentin 蛋白表达明显不同，并且我们还对 Vimentin 表达作了更深入的研究。所得数据显示，其异质性也存在于单一克隆内部以及自身在放射处理前后的时间跨度上。这另一方面反映出许多肿瘤细胞在临床治疗干预下，会更多地被诱导产生如表皮 - 间质化等时间异质性的转变，导致其侵袭能力、远端转移能力的变化[27,28]。

此外，与单克隆 3 相比，具有高表达 Vimentin 蛋白的单克隆 1 的确有着较强的侵袭能力，而单克隆 2，虽然 Vimentin 蛋白处于极低表达状态，但依然也能表现出相当强的侵袭力。这些结果提示 Vimentin 的表达并不是判断肿瘤侵袭力的唯一金标准，影响 Vimentin 蛋白表达的强弱因素有很多易变环节[11,26]，如何寻找可靠的相关指标也对研究者提出更高要求[28-30]。因此，许多临床鉴定追踪特定肿瘤细胞群体时，往往依靠单一的生物识别标记并不能以偏概全，肿瘤细胞的这种异质性需要复合标记物的识别[31,32]，从而达到临床个体化、精准治疗的需求。

总之，肿瘤细胞自身异质与时间异质性往往会随其进展、治疗干预手段的不同，呈现出不同的时相阶段[18,33,34]。如何更好理解与研究肿瘤细胞的基础生物学特性，对于指导临床治疗手段以及对应窗口期的把握是至关重要的。

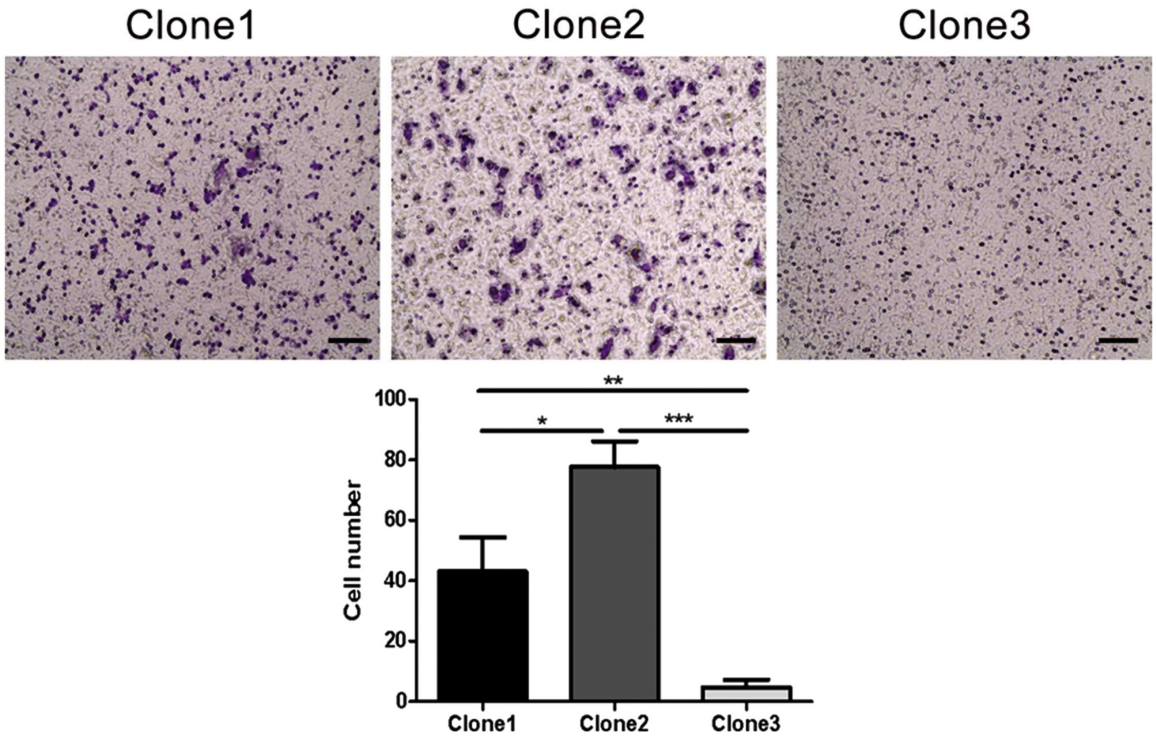

图 5 克隆 1、克隆 2 和克隆 3 的侵袭力差异 (放大倍数:× 200)

Fig.5 Diversity of invasiveness of clone1, clone2 and clone3 (Magnification: × 200)

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