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Ⅳ型胶原在癌侵袭转移过程中的动态变化：脉冲模式 *

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摘要 结合量子点原位分子成像技术探究了基于Ⅳ型胶原动态改变的癌侵袭转移模式．收集肝癌、胃癌、乳腺癌和宫颈癌的临床病理标本，利用免疫组化和量子点成像技术对癌细胞及其相关微环境中的关键分子进行成像，观察癌侵袭转移过程中Ⅳ型胶原的动态变化．结果表明，在癌侵袭和转移过程中Ⅳ型胶原呈现动态改变．首先在基底膜处交联增多，形成不规则且致密的袖套包裹癌巢；随后多处基底膜处的胶原发生构象改变并被降解形成侵袭前锋．同时，伴随着间质中的Ⅳ型胶原重新线性沉积及巨噬细胞的团聚增多，癌细胞最终逃逸转移．由上述结果可以断定，癌侵袭转移呈现“脉冲模式”，Ⅳ型胶原的动态改变为癌侵袭转移创造了适宜的微环境．

关键词 癌，Ⅳ型胶原，细胞外间质重塑，量子点
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癌症是世界上首位致死病因，每年有 760 万人死于癌症[1]，癌症致死的主因是侵袭和转移[2]，二者是癌的最本质特征[2]．然而，导致癌侵袭和转移的根本机制尚不明确．20 世纪的癌症研究主要关注癌细胞自身，部分阐释了癌发生、发展的遗传学及表观遗传学机制[3-6]．但本世纪学界逐渐认识到，必须将癌细胞放在特定的环境中进行研究，才能得到更合乎临床实际的信息[7-9]，因此，充分挖掘隐藏在肿瘤微环境中的丰富信息，有助于全面认识癌侵袭转移行为．

肿瘤微环境中最值得关注的是细胞外间质(extracellular matrix，ECM)．在癌侵袭过程中，ECM 发生了重要的结构重塑，主要特征是纤连蛋白、蛋白聚糖类和胶原沉积增加，基质交联增强[10-11]．其中，Ⅳ型胶原是基底膜的主要成分，主要位于上皮和内皮细胞的基底层，为 ECM 成分提供支架，因此破坏Ⅳ型胶原成为癌侵袭转移的先决条件，该过程必须依赖于基质金属蛋白酶(matrix metalloproteinases，MMPs)[12]．通过胶原降解而形成潜在的癌细胞逃逸通道[12]、扰乱肿瘤微环境增加机械张力以诱导上皮间质转化[13]，暴露胶原活性位点募集单核细胞以激发免疫应答级联反应[14]，直接启动癌细胞增生、侵袭和血管形成[15]．Ⅳ型胶原三螺旋结构的直径是 15 nm，而 MMPs 中起催化作用的结构域只有 5 nm[15]．在被 MMPs 降解之前，Ⅳ型胶原先要经历一系列结构改变，即所谓的分子构造学[16]．因此，Ⅳ型胶原的动态改变是探索基于 ECM 层面认识癌侵袭机制的前沿，迫切需要新技术显示动态过程．

量子点(quantum dots，QDs)是具有独特光学性质的纳米粒子，在肿瘤学研究中有很好的前景[17]．相比于传统的有机染料和荧光蛋白，量子点有诸多优势特征，如具有较好的可调控性激发光、较强光亮度及抗猝灭能力[18-19]．本研究采用基于量子点标记分子探针的原位分子成像技术，研究癌侵袭过程中包裹癌巢的Ⅳ型胶原的改变．

1 材料与方法

1.1 临床病理切片收集和处理

临床石蜡病理切片来自武汉大学中南医院和武

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汉市中心医院，包括肝癌、胃癌、乳腺癌和宫颈癌．石蜡切片 4 μm 厚连续切片，60℃ 烤片 2 h，在二甲苯中脱蜡 3 次，每次 5 min．无水乙醇浸泡 5 min，然后再分别用 95%乙醇浸泡 2 次，每次 2 min，85%乙醇 1 次，2 min 和自然水冲洗 5 min，最后用蒸馏水冲洗 3 min．然后切片置于 0.01 mol/L 柠檬酸盐缓冲液(pH 6.0)，微波 98℃ 修复 20 min．在室温下冷却 30 min，之后用蒸馏水冲洗．

1.2　免疫组化

用 3% H_2O_2 在室温下孵育 10 min，消除内源性过氧化物酶．每张切片用 2% BSA 37℃ 封闭 30 min，防止抗原与抗体非特异性结合．然后倾去 BSA，不用冲洗，滴加一抗，在 37℃ 孵育 2 h．一抗分别是Ⅳ型胶原(ab-6586，Abcam，英国，稀释比 1/300)、巨噬细胞(MA1-38069，ABR，美国，稀释比 1/300)和 Ki67(MAB-0129，Maxim-Bio Co，中国，工作液)．然后用 TBS 冲洗 3 次，每次 5 min，滴加相应二抗，37℃ 孵育 15 min，最后用过氧化物酶标记的链霉素孵育 15 min(福州迈新生物技术开发有限公司，中国)．再用 PBS 冲洗 3 次，每次 5 min．每张切片滴加新鲜配制的 DAB 溶液显色(DAKO，丹麦)，镜下观察显色反应，显色适度时终止反应．自来水充分冲洗，用苏木素复染，封片．阴性对照组的一抗用 TBS 替代．

1.3　量子点染色

一抗是兔抗人Ⅳ型胶原多克隆抗体(ab-6586，Abcam，英国，稀释比 1/300)．二抗是山羊抗兔抗体 QDs-585(Invitrogen，美国，稀释比 1/400)．用 2% BSA 封闭后，滴加一抗 37℃ 孵育 2 h．用 PBS 冲洗 3 遍后，再用 2% BSA 37℃ 封闭 30 min，再加 QDs 二抗 37℃ 孵育 2 h．冲洗后即可观察．

1.4　图像获取与分析

所有切片均在带有 Olympus BX51 的荧光显微镜下观察．QDs-585 用紫外(UV，330～385 nm)激发，所有图像均由 DP72 镜头获取．基于上述所获取的图片，分析Ⅳ型胶原在肝癌、胃癌、乳腺癌和宫颈癌侵袭前锋及周围间质中的不同变化特征，研究Ⅳ胶原在宫颈癌与胃癌不同进展阶段的动态变化特点．

2　结果与讨论

2.1　Ⅳ型胶原在癌组织中的特点与分型

Ⅳ型胶原是 ECM 最主要的成分，在不同癌组织中，Ⅳ型胶原的形态学及分布具有明显的异质性，但主要有以下 4 个特点：a．乳腺癌组织中的Ⅳ型胶原通常以双层结构包裹癌巢外周，并且内层比较模糊，而当有癌细胞向外突出侵袭至间质时，外层胶原呈现不连续，形成了所谓的癌侵袭前锋(图 1a)；b．胃高分化管状腺癌中的Ⅳ型胶原主要展现出碎片状及扭曲的双层或多层结构．许多细小的碎片随机播撒在癌巢周围的肿瘤间质中，形成一个看似混乱的场面(图 1b)；c．宫颈癌中的Ⅳ型胶原是以疏松和分层的方式包裹癌巢，并且伴随一些模糊的碎片状胶原无序散布在基底膜处(图 1c)；d．肝内高分化胆管癌的Ⅳ型胶原在癌侵袭前锋为双层结构，在癌巢交界融合处胶原特别丰富(图 1d)．

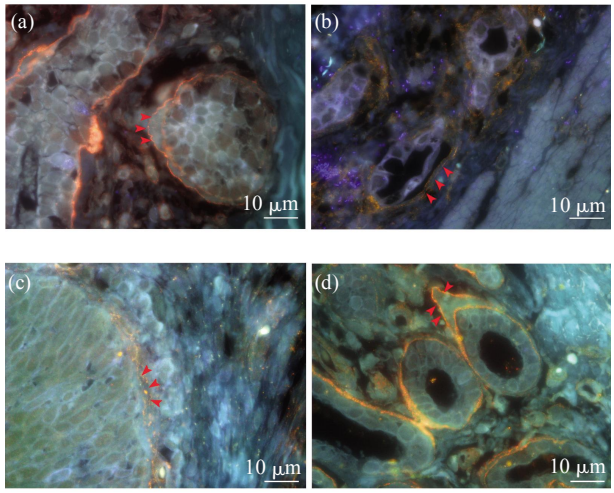

Fig. 1　The morphological changes of collagen Ⅳ in cancer tissues based on QDs-585 molecular imaging

(a) Double-layer collagen Ⅳ surrounds breast cancer nests with fuzzy inner layer compared with outer layer, especially at invasion front. (b) Double-layer collagen Ⅳ surrounds gastric cancer (GC) nests with irregular deposition in ECM. (c) Collagen Ⅳ surrounding cervical carcinoma (CC) nests is loose with multiple-layer. (d) Collagen Ⅳ in intrahepatic biliary carcinoma nests presents double-layer at invasion front. (Red arrow heads indicate the sites with collagen Ⅳ changes).

上述Ⅳ型胶原在不同癌组织中呈现出的异质性变化，可分成 4 类(图 2)：a．线性．Ⅳ型胶原在间质中呈现僵直形态紧密包绕癌巢，促使癌巢内张力增加．b．不规则型．不规则的弯曲型胶原主要围绕癌巢周围，并包裹着癌巢．c．碎片型．在基底膜处线性及不规则型胶原被降解，伴随着间质碎片状胶原增多，弥散分布．d．缺失型．在癌巢中及其周围间质中均无Ⅳ型胶原．上述Ⅳ型胶原的改变，从基底膜丰富型至间质中丰富型，最终至基底膜及间质中均缺失型，提示Ⅳ型胶原的不同类型可能与肿瘤的侵袭模式相关[20]．

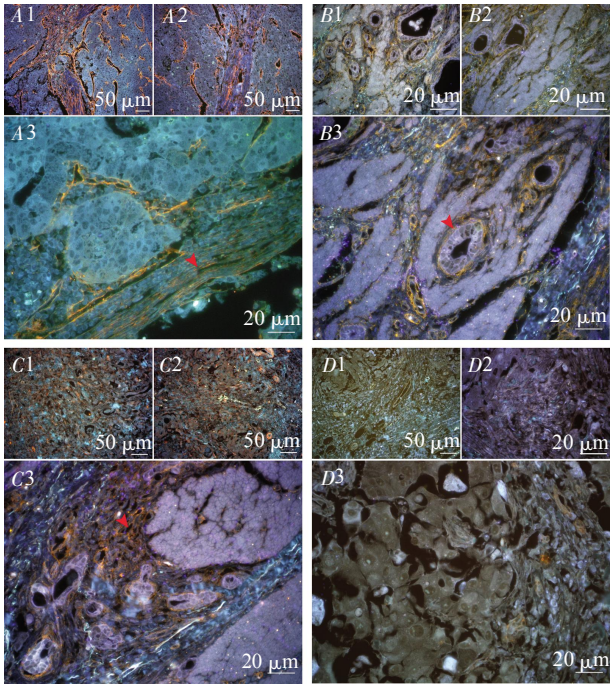

Fig. 2　Typical collagen Ⅳ alterations in different cancer tissues

*A*1～*A*3 show linear type of collagen Ⅳ aligned with hepatocellular carcinoma nests. *B*1～*B*3 show irregular type of collagen Ⅳ surrounding GC nests. *C*1～*C*3 show fragmented type of collagen Ⅳ depositing in ECM of GC. *D*1～*D*3 show collagen Ⅳ disappears both in ECM and around tumor nests. Red arrow heads indicate the sites with collagen Ⅳ changes.

2.2　鳞癌侵袭过程中的Ⅳ型胶原动态变化：以宫颈癌为例

对宫颈癌及其癌旁组织中 3 个主要成分进行多分子成像研究，揭示癌侵袭过程中的关键事件：反映癌细胞增殖指数的 Ki67，反映免疫浸润的巨噬细胞以及反映间质重塑的Ⅳ型胶原(图 3)．在癌旁组织(图 3*A*1)中，Ki67 阳性细胞仅分布在表皮基底细胞层(图 3*A*2)，而宫颈癌组织(图 3*B*1)中 Ki67 阳性细胞弥散分布在整个癌巢中(图 3*B*2)．同样地，在癌旁组织中巨噬细胞以单个形式分散在基底膜下间质中(图 3*A*3)，而在癌组织中巨噬细胞却以团聚形式分散在癌细胞间及其间质中(图 3*B*3)．结合传统的免疫组化及量子点成像技术，清晰显示Ⅳ型胶原在宫颈癌组织中的动态变化过程．在癌旁组织中，Ⅳ型胶原在基底膜处呈现一条光滑、完整的线条(图 3*A*4～*A*6)．而在癌组织中，该光滑且完整的线变成多层不清晰的线条，并行包绕着癌巢(图 3*B*4 和 *B*7)．随后，多层不清晰的线条状胶原变得更加疏松且模糊(图 3*B*5)，尤其以癌细胞凸向间质处为明显，且周围间质中Ⅳ型胶原的沉积增 加并伴有血管生成(图 3*B*6 和 *B*8, *B*9)．Ⅳ型胶原在宫颈癌中呈现动态变化过程，提示其在肿瘤进展中扮演的角色，不仅仅是传统意义上的机械屏障[21].间质中沉积的胶原及其在包裹癌巢的基底膜处发生的由单层至多层的改变，均提示Ⅳ型胶原的动态变化参与了肿瘤从原位向间质浸润的过程[22]．并且，基底膜处胶原由多层致密变为模糊疏松，同时伴有邻近间质中胶原沉积，提示基底膜处胶原降解及其间质中胶原活性肽产生．该过程暴露了不同活性的位点，进一步诱导肿瘤新生血管生成[11]，募集单核细胞诱发级联免疫应答反应[23]，这也与我们的结果显示的巨噬细胞在癌组织间质中显著增加相一致．

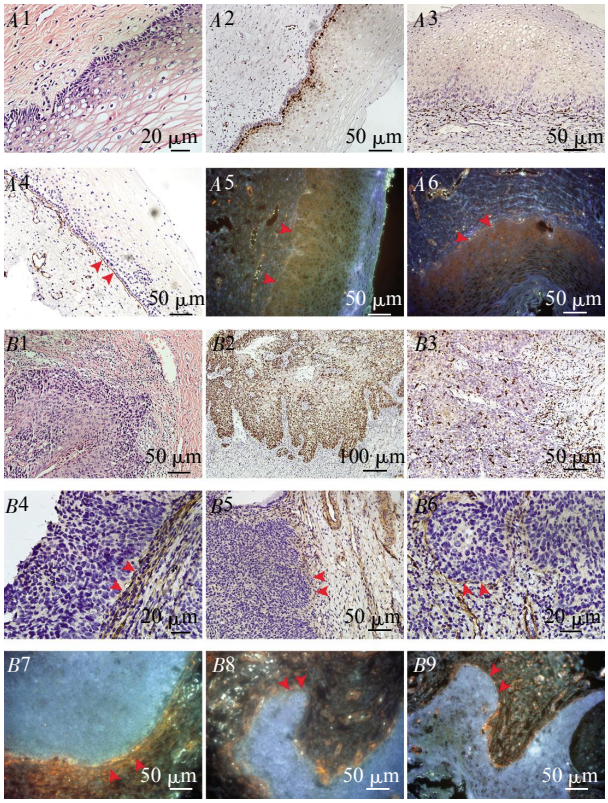

Fig. 3　Comparison between CC tissue and peri-tumor tissues based on multiple molecular biomarkers

*A*1, *B*1 show HE staining of peri-tumor tissue and CC tissue respectively. *A*2, *A*3 show expressions of Ki67 and macrophages in peri-tumor tissues by immunohistochemistry (IHC), compared with those in CC tissue (*B*2, *B*3 respectively). *A*4～*A*6 show collagen Ⅳ expression in peri-tumor tissues, presenting an intact and smooth line both by IHC (*A*4) and QDs-585 (*A*5, *A*6). *B*4, *B*7 show increased collagen Ⅳ deposition forming dense multi-layer aligned with CC nests by IHC and QDs imaging, respectively. *B*5 shows loose and fuzzy layers of collagen Ⅳ surrounding CC nests. *B*6, *B*8, *B*9 show cancer cells protrude into stroma with disappearance of loose collagen Ⅳ. Red arrow heads indicate the sites with collagen Ⅳ changes.

2.3　腺癌侵袭过程中的Ⅳ型胶原动态变化：以胃癌为例

在胃高分化管状腺癌侵袭过程中，Ⅳ型胶原与癌巢之间呈现一个动态演变过程．在癌侵袭的早期阶段，随着癌巢的扩张，包裹癌巢的Ⅳ型胶原逐渐增多[24]，限制癌细胞向外逃逸(图 4a, b)．同时，Ⅳ型胶原经历着一系列如交联及解螺旋等[16]的结构改变，与癌巢并行排列的Ⅳ型胶原逐渐变成一个复杂的双层甚至多层结构，为Ⅳ型胶原的降解提供场所[11]．当具有侵袭行为的癌细胞群体形成侵袭小癌巢时，围绕其表达的Ⅳ型胶原被完全降解消失(图 4c, d)．同时，这种在癌侵袭前锋部位的Ⅳ型胶原降解伴随着间质中不规则碎片状Ⅳ型胶原的沉积增加．间质中Ⅳ型胶原的上述改变可以扰乱间质内环境稳态[25]，增加血管生成[26]，并且诱导免疫应答以利于癌侵袭转移[27]．

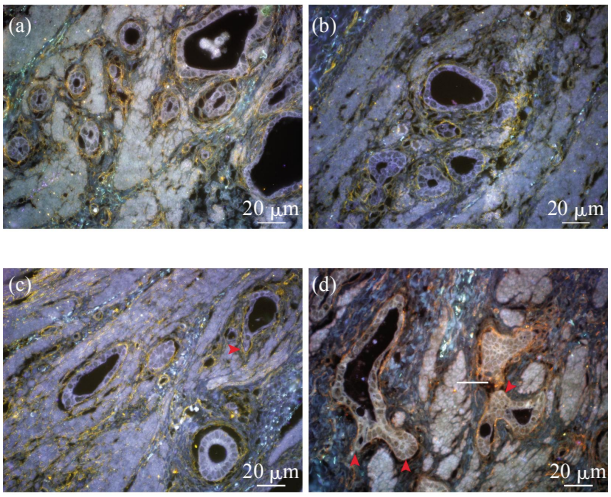

Fig. 4　Collagen Ⅳ changes of GC during invasion process based on well-differentiated tubular adenocarcinoma

(a, b) show GC nests are surrounded by intact and dense collagen Ⅳ almost without degradation and ECM deposition. (c, d) show collagen Ⅳ aligned with GC nests is degraded at invasion front (Red arrows) with increased irregular collagen Ⅳ deposition in ECM.

2.4　癌侵袭模式的预测：基于Ⅳ型胶原降解及重塑的动态演变

肿瘤侵袭是一个多因素、多阶段、多环节、逐渐发展的过程，涉及多种基因的异常表达及多条信号通路的激活，最终导致肿瘤微环境紊乱，使其向有利于肿瘤侵袭转移的方向发展[28]．肿瘤微环境的时空改变及其与癌细胞的协同进化过程，在调节诱导癌细胞休眠、增生、侵袭及转移等过程中均起关键作用[9]．肿瘤微环境的重要性备受关注，包括ECM 及基质硬度在细胞极性及组织生长潜力中所扮演的角色，细胞外生长因子及基质分子在癌侵袭转移中所起的作用，其中尤以Ⅳ型胶原的作用最为显著[10]．

Ⅳ型胶原是细胞外间质中最主要的成分之一，主要在基底膜处表达．随着癌细胞增生，肿瘤内出现中心缺氧，刺激癌细胞分泌赖氨酰氧化酶(lysyl oxidase，LOX)，后者可激活基底膜处Ⅳ型胶原的级联交联反应[11]，形成一个不规则且致密的袖套包裹癌巢，胶原呈现双层化和多层化改变．随着癌巢增大，癌巢内的张力增加，最终导致癌细胞向外浸润以减轻癌巢内的压力．在侵袭前锋的局部，癌细胞团分泌 MMPs 降解Ⅳ型胶原，同时多处基底膜的胶原呈碎片状，发生解螺旋等构象改变以便被进一步降解．侵袭前锋的胶原消失，癌细胞从胶原降解处逃逸，形成新的小癌巢．另外，降解的胶原变成胶原肽，随后在间质中重新沉积形成线性胶原，使间质的胶原增多并显得僵硬，增加肿瘤间质的硬度，增加癌巢周围间质张力，进一步激活整合素[29]，驱动分子黏附过程[30]，影响黏着斑形成[31]并增加黏着斑激酶活性[32]，最终促进癌细胞增生、侵袭、转移及肿瘤血管生成[13]．

上述基底膜处Ⅳ型胶原先增多后减少至最终消失，及间质中的Ⅳ型胶原线性沉积及线性化等的一系列改变，最终形成了肿瘤进展过程中的关键“预转移场”，导致癌细胞群体从大癌巢中逃逸，形成独立的小癌巢．释放出来的小癌巢又会重复上述肿瘤进展过程．根据我们的研究结果，结合文献报道，我们提出肿瘤浸润进展的“脉冲模式”(图 5)，认为在肿瘤侵袭进展过程中，当癌细胞及其微环境的共同演变至一定程度，细胞外间质张力持续增加，达到癌侵袭转移“关键临界点”，癌组织中的大癌巢即会发生“爆炸”，降低细胞外间质张力并播撒很多“种子”小癌巢，种植在这块肥沃的“土壤”里，即所谓的功能紊乱的肿瘤微环境．这些小的种子癌巢则会重复下述过程：癌细胞增生 - 中心缺氧区形成 - 癌巢基底膜处Ⅳ型胶原交联及沉积 - 癌巢外间质重塑 - 癌巢内张力增大 - 基底膜处胶原降解 - 癌巢爆炸．该理论的潜在意义在于：a．研究降低癌巢内的张力策略，延缓或阻滞癌巢“爆炸”，可能有助于防治癌侵袭转移；b．癌症治疗中除了重点降低癌细胞增生活性外，还要关注调控肿瘤微环境．

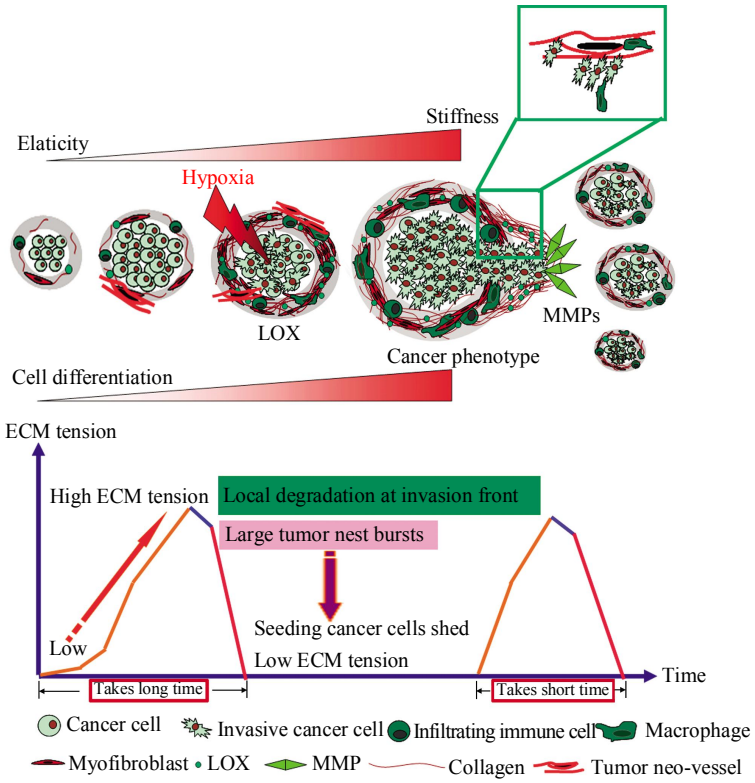

Fig. 5　Pulse-mode of cancer invasion and metastasis

As tumor mass expands, ECM remodeling occurs characterized by collagen Ⅳ changes including degradation and re-deposition. Prominent central hypoxia cancer cells secret LOX to cross-link collagen Ⅳ to constrain cancer cells and subsequently to be degraded followed by increasing collagen Ⅳ deposition in ECM. Tumor mass becomes increasingly harder with tumor stroma stiffening causing high ECM stress. With cancer cells proliferation, tension becomes increasingly higher, till reaching a critical point, where large tumor nests "burst", releasing many tiny seeding nests and reducing the central ECM tension. The resulting seeding tumor nests repeat the same process of tumor mass growth→prominent central hypoxia→collagen Ⅳ cross-linking and deposition→ECM remodeling→ECM stress buildup→tumor nest bursts, leading to accelerated cancer progression in this rich "soil" of dysfunctional tumor microenvironment.

因此，在肿瘤进展过程中，认识Ⅳ型胶原改变及 ECM 重塑有重要意义．下一步工作是解析Ⅳ型胶原的构象，探索其三螺旋结构的转化过程，及调控癌细胞侵袭行为的分子基础．

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Dynamic Changes of Collagen Ⅳ During Cancer Invasion and Migration: Pulse Mode*

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Abstract Cancer invasion and metastasis remain two root causes of mortality. This process involves alterations of tumor microenvironment, particularly the remodeling of extracellular matrix(ECM), characterized by collagen Ⅳ uncoiling, degradation, fragments deposition and cross-linking. This study was aimed to reveal the cancer invasion mode based on dynamic changes of collagen Ⅳ by novel quantum dots (QDs) imaging technology. Cancer tissues of hepatocellular carcinoma, gastric cancer, breast cancer and cervical carcinoma were collected and stained by traditional immunohistochemistry and novel QDs-based imaging technology. Several key molecules representing of cancer cells and tumor microenvironment were studied, including Ki67, representing of proliferation of cancer cells, macrophages, representing of monocytes infiltration and collagen Ⅳ, representing of tumor stroma remodeling during cancer invasion and migration. During cancer invasion and migration, collagen Ⅳ had structural and functional changes. In different cancer tissues, 4 types of collagen Ⅳ could be observed, consisting of linear, irregular, fragmented and disappeared. However, several common features were evident during the dynamic process of cancer progression. First, collagen Ⅳ at basement membrane increased, presenting an irregular sheath surrounding cancer nests; then, collagen Ⅳ was degraded to form invasion fronts at several sites accompanied with linear re-deposition of collagen Ⅳ and increased macrophages in ECM. Second, cancer cells escaped from large cancer nests to seed in ECM. And also the dynamic changes of collagen Ⅳ were accompanied with the recruitment of macrophages, together to regulate and affect biological behaviors of cancer cells. A series changes caused by dynamic changes of collagen Ⅳ provide a proper tumor microenvironment for cancer invasion and migration. In this dysfunctional tumor microenvironment, tumor mass becomes increasingly harder with tumor stroma stiffening causing high ECM stress. With cancer cells proliferation, tension becomes increasingly higher, till reaching a critical point, where large tumor nests "burst", releasing many tiny seeding nests and reducing the central ECM tension. The resulting seeding tumor nests repeat the same process of tumor mass growth→prominent central hypoxia→collagen Ⅳ cross-linking and deposition→ECM remodeling→ECM stress buildup→tumor nest bursts, leading to accelerated cancer progression in this rich "soil" of dysfunctional tumor microenvironment, presenting a "pulse mode".

Key words cancer, collagen Ⅳ, ECM remodeling, quantum dots

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