以微小RNA作为新型标志物检测循环肿瘤细胞及其临床意义

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摘要 循环肿瘤细胞(circulating tumor cell, CTC)是随血液循环一起转运的实体肿瘤细胞, 与实体肿瘤的发展、转移、复发和预后等关系密切。然而, CTC数量的稀少使有效检测CTC具有较大的挑战性。微小RNA(microRNA, miRNA)作为一类新发现的基因表达调控分子, 在肿瘤的发生、发展、转归等过程中起着重要的作用。CTC关联性miRNA的研究为CTC的检测和肿瘤的诊治开创了新思路。该文介绍了CTC的临床意义和主要分析方法, 在CTC关联性miRNA与肿瘤诊断、治疗和预后等方面总结了这类新型肿瘤细胞标志物的研究进展。

关键词 微小RNA; 循环肿瘤细胞; 肿瘤标志物; 细胞分析

1 引言

目前, 肿瘤的发病率和死亡率持续攀升已成为当今社会的重要问题之一[1]。一般认为, 肿瘤的早期发现对于提高肿瘤的诊治水平和改善患者的预后具有积极意义, 而现阶段对绝大多数肿瘤的诊断主要依赖于内镜、组织活检和物理诊断等方法, 这些方法对于肿瘤的早期诊断效果不佳。

实体瘤患者血液中存在的循环肿瘤细胞(circulating tumor cell, CTC)不仅为肿瘤的诊断及其转移的判断提供了客观依据, 而且还被认为是一种极具潜力的治疗靶点[2]。由于微小RNA(microRNA, miRNA)与肿瘤发生和发展的关系非常密切, 有希望成为肿瘤诊断的新型标志物和治疗的新靶点[3-5], 因而CTC关联性miRNA的分析在肿瘤诊治中的意义日益呈现。为此, 本文综述了这方面的最新研究进展。

2 CTC概述

2.1 CTC及其临床意义

从肿瘤原发灶或转移灶脱落入血, 并随机体血液循环一起转运的实体肿瘤细胞, 被称为循环肿瘤细胞(CTC)。脱落到循环血中的癌细胞具有恶性肿瘤细胞的内在生物学特性, 是了解肿瘤转移情况和制定合理治疗方案的参考依据[6-7]。了解CTC与肿瘤存在、转移和预后的关系具有较为重要的临床意义。

2.1.1 CTC与肿瘤的存在 CTC的发现意味着肿瘤的存在。CTC被认为是实体肿瘤的早期事件, 检测到CTC的实体瘤患者应按高危人群进行动态观察, 并给予相应的治疗[6-7]。有研究发现, 结肠癌术后患者中约有45%的患者死于远处转移[8]。究其原因, 术后残留CTC的存在为罪魁祸首[8]。CTC数量稀少, 特别是在肿瘤的早期阶段, 其数量更为稀少[9]。因此, 改进CTC的检测方法、探寻理想的CTC标志物, 对于早期发现肿瘤的存在具有非常重要的意义。

2.1.2 CTC与肿瘤的转移 CTC与肿瘤转移的关系非常密切[6]。在转移性乳腺癌的相关研究中, 人们应用CTC分析系统(CellSearch™, 美国Veridex LLC公司生产)、免疫组织化学等方法发现了患者外周血中存在明显的CTC[6,10-11]。局灶性前列腺癌患者血象中也发现了CTC, 而且在有转移的患者中, CTC的发现率相对更高, 提示CTC的检测量可能与肿瘤的进程呈正相关[12]。Armstrong等[13]也认同上述观点, 并认为兼具上皮型(epithelial phenotype)和间质型(mesenchymal phenotype)的CTC可能为过渡型细胞, 即上皮间质转化(epithelial-mesenchymal transitions, EMT)型细胞, 而这类细胞与肿瘤转移和侵袭密切相

收稿日期: 2012-04-19 接受日期: 2012-06-18

国家自然科学基金(No.81171660)、浙江省公益类科技攻关计划(No.2010C33112)、宁波市自然科学基金(No.2010A610044)、浙江省新苗人才计划(No.2010R405059)、宁波市科技创新团队项目(No.2011B82014)、宁波市重点学科项目(No.XKL11D2127, No.XKL11D2128)和宁波大学实验技术研究开发项目(No.SYJS-201103)资助项目

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关。类似的研究还发现, 结肠癌患者CTC的检出率与结肠癌的分期呈正相关, CTC数量多者其转移潜能也大[9]。Nakagawa等[14]发现, 血象中存在CTC的乳腺癌患者具有较高的腋前淋巴结转移的风险, 因而CTC可作为评估是否具有乳腺癌淋巴结转移的指标之一。

2.1.3 CTC与肿瘤的预后 CTC对于判断肿瘤患者的预后也有重要价值[6]。Tsouma等[8]发现, CTC的水平与肿瘤患者分期密切相关, 早期发现CTC有利于改善患者的预后。Giuliano等[15]在研究CTC检出率与乳腺癌患者疾病进程和生存率的关系时发现, 治疗前CTC的数量可作为转移性乳腺癌无进展生存时间(progression-free survival, PFS)和总生存期(overall survival, OS)的一项独立预测指标。对于早期肿瘤患者, CTC数量的升高往往意味着不甚乐观的预后; 同样, 对于进展期肿瘤患者则预示着PFS和OS的降低。CTC的丰度已被证明与肿瘤患者OS缩短相关, 如应用于肿瘤患者的诊治过程中可提高对患者预后评估的准确性[16]。Poveda等[17]在研究晚期复发性卵巢癌的PFS和OS时发现, CTC数量的增多预示着卵巢癌的不良预后。因此, CTC的数量与肿瘤患者的预后呈负相关。

2.2 CTC分析的主要方法

分析CTC的方法主要包括形态学、免疫学、细胞学和分子生物学等[18]。这些方法的检测原理各异, 也各有优缺点(表1)。CellSearch™和CTC芯片均为基于免疫学的CTC检测方法, 它们既可富集又可检测CTC, 但只能检测上皮黏附分子(epithelial cell adhesion molecule, EpCAM)阳性的CTC。基于形态学的上皮肿瘤细胞大小分选法(isolation by size of epithelial tumor cell, ISET)、密度梯度(density gradient)及改进的带有多空筛离心管的Oncoquick(porous barrier separates density gradient)富集法均可检测EpCAM阴性的CTC。Hofman等[19]发现, 联合使用CellSearch™和ISET可提高CTC的检出率。联合密度梯度分选及抗体介导的RARE(RosetteSep-applied imaging rare event)富集法通过排除血样中的大部分红细胞和白细胞, 可应用于CTC的富集; 而基于联合使用免疫磁珠与实时定量反转录聚合酶链式反应(reverse transcription-polymerase chain reaction, RT-PCR)技术的AdnaTest(德国AdnaGen公司产品)方法具有较高的敏感性和特异性。免疫磁珠分选系统(magnetic activated cell sorting system, MACS)是基于免疫标记的CTC富集方法, 其重复性和精确性较好。基于细胞计数的CTC检测法如光纤阵列扫描技术(fiber-optic array scanning technology, FAST)和激光扫描细胞术(laser scan cytometry, LSC)无需先行CTC的富集; 而上皮免疫斑点法(epithelial immunospot, EPISPOT)可检测具有活性的CTC。

CTC富集法中, ISET、RARE和MACS虽然有较好的敏感性和特异性, 但并无大规模病例研究的报道; 同样, EPISPOT和FAST也并无较多明确的临床相关性的研究结论[20]。CellSearch™和RT-PCR技术的应用近年来相对较广。Gervasoni等[21]发现RT-PCR技术在提高结肠癌CTC检出率上具有灵敏度高的优点。RT-PCR方法在评估CTC时更为客观化的优势引起了人们的关注[22]。van der Auwera等[23]在比较CellSearch™、AdnaTest和RT-PCR三种方法用于转移性乳腺癌CTC检测时发现, 多标志物RT-PCR检测比前二者有更高的敏感性。Chen等[24]也发现, 标志物的联合应用可提高CTC检测的效力。因此, 筛选合适的CTC标志物在CTC检测中具有重要的意义。

3 CTC关联性miRNA在肿瘤诊治中的临床意义

miRNA是一类在进化史上极为保守的内源性非编码小RNA, 它们通过诱导目标mRNA的降解或干扰蛋白质的翻译过程下调特异性基因的表达, 在控制细胞的生长、分化和凋亡等方面起着非常重要的作用[25-27]。研究表明, 许多miRNA与肿瘤(包括实体瘤)的发生和发展有重要的关系, 它们扮演着癌基因或抑癌基因的角色[26]。作为基因的直接转录本, 具有与肿瘤密切关联的特性赋予了一些miRNA成为CTC新型标志物的潜力[28-29]。尽管在肿瘤患者的血浆或血清中也可以检测到异常水平的miRNA, 但因血中游离性miRNA的来源和去路的机制尚未完全清楚[7], 而且有研究证明正常人血象中也存在至少100种miRNA[30], 所以, CTC关联性miRNA因更能表现肿瘤负荷和体现上皮细胞特性, 正在展示其在实体肿瘤的转移监测、预后判断和治疗选择等方面的巨大潜力。

3.1 CTC关联性miRNA与肿瘤的诊断

miRNA在生物体呈现高度的特异性, 赋予了其作为诊断学证据的价值。Taylor等[31]研究发现, 在卵

表1 循环肿瘤细胞检测的常用方法

Table 1 The common methods for the detection of circulation tumor cells

名称 Names	原理 Principle	优点 Advantages	缺点 Disadvantages	灵敏度 Sensitivity
ISET	Size discrepancies between CTC and normal blood cells	Widely used for various types of tumors, easy and rapid, without damaging CTC's morphology, feasible for EpCAM negative CTCs, high sensitivity	Low specificity, loss of CTC smaller than the filter pore, retain large leukocytes	1 cell/mL
Density gradient separation	Density discrepancies among different elements of blood	Easy and inexpensive, feasible for EpCAM negative CTCs	Low specificity, larger blood volume	Unclear
Oncoquick	Density discrepancies with porous barrier	Easy and inexpensive, feasible for EpCAM negative CTCs, less cross contamination	Low purity, low specificity	1 cell/4.6 μL
MACS	Immuno labeled magnetic microbeads	Isolate CTCs with its integrity preserved, multisorting strategy, high reproducibility and accuracy	Possible false-positive or false-negative results	1 cell/0.3 mL
CellSearch	Capture of CTCs by EpCam labeled ferro fluid	High sensitivity and specificity, automated, quantitative, highly reproducible, moderate blood volume required, commercially available, only assay approved by FDA	Only EpCAM-positive CTCs can be fished out, possible false-positive or false-negative results, no further analysis possible	1 cell/0.5 mL
RARE	Combinative use of density gradient separation and antibody	Low contamination from white blood cell, widely used for various types of tumor	Non-automated	Unclear
AdnaTest	A combination of immuno-magnetic separation and RT-PCR assay	High sensitivity and specificity recognition of tumor specific markers (EpCAM, MUC1), downstream tumor associate genes analysis	No flexibility, possible false-positive or false-negative results	1 cell/2.5 mL
CTC Chip	Capture of CTCs by EpCAM-coated microposts under strict manipulation of velocity and shear force	Isolation of viable cells (viability 98%), high sensitivity and specificity, minimal processing of samples, potential to harvest CTCs for further molecular and genetic analyses	Only EpCAM-positive CTCs can be fished out, possible false-positive or false-negative results, not commercially available, no validation studies in large scale clinical settings	1 cell/10^9 blood cells
EPISPOT	Based on ELISA	Isolation of viable cells and secretory proteins from CTCs	The secretory proteins from CTCs should have activity	Unclear
FAST	Wide-field and digital microscope	Continuous scanning, no need to enrich	Subjective interference	Unclear
LSC	Using laser-based opto-electronics and automated analysis capabilities	No need to enrich	Subjective interference	Unclear
RT-PCR	Amplification of the targeted RNA	High sensitivity, rapid, quantitative, small sample volume required	Technique issue with RNA degradation, false-positive or false-negative results, no distinction between viable and non-viable cells, no visualisation of CTCs, no further analysis possible	Unclear

ISET: 上皮肿瘤细胞大小分选法; MACS: 免疫磁珠分选系统; RARE: 使用RosetteSep成像稀少事件; CTC: 循环肿瘤细胞; EPISPOT: 上皮免疫斑点法; ELISA: 酶联免疫吸附法; FAST: 光纤阵列扫描技术; LSC: 激光扫描细胞术; RT-PCR: 反转录–聚合酶链式反应。

ISET: isolation by size of epithelial tumor cells; MACS: magnetic activated cell sorting system; RARE: RosetteSep-applied imaging rare event; CTC: circulating tumor cell; EPISPOT: epithelial immunospot; ELISA: enzyme-linked immunosorbent assay; FAST: fiber-optic array scanning technology; LSC: laser scan cytometry; RT-PCR: reverse transcription-polymerase chain reaction.

巢癌患者CTC中检测到8种过表达的miRNA(miR-21、 miR-141、miR-200a、miR-200c、miR-200b、miR-203、

miR-205和miR-214), 患者CTC中CTC关联性miRNA与普通细胞的表达水平差异达到2~7倍不等; 他们还证实了以这些miRNA作为标志物在替代组织活检应用于无症状人群的筛查中具有一定的意义, 提示这种更为便捷和微创的卵巢癌筛查方法具有较大的推广价值。在本实验室先前的研究中, 我们发现胃癌组织和癌旁组织有19种差异表达的miRNA[32]; 进一步研究发现, 它们当中的miR-106a和miR-17在胃癌患者外周血有核细胞中的水平明显高于健康对照, 分别平均增加37.32倍和36.95倍, 而且术前患者的水平明显高于术后患者的水平, 提示二者具有应用于CTC检测的潜力。对倍稀释胃癌SGC-7901细胞的回收实验结果证实, 以miR-106a和miR-17作为标志物用于检测CTC的灵敏度达到1个CTC细胞/mL血液[33]。进一步临床分析发现, 采用RT-PCR检测胃癌CTC相关性miR-106a和miR-17的截断值分别为6.54和6.19[以阈值循环(threshold cycle, C_t)表示]。另外, 由于这两种miRNA的诊断价值相似, 联合使用不能提高检测CTC的效力[33], 而其他小RNA的联合使用却可以提高检测CTC的效力[7]。国外有研究发现, 与健康人和未能检测到CTC的患者相比, 从不少于5个CTC中就可检测出10种高表达的肿瘤相关miRNA[20]。最近, 我们在检测CTC关联性miR-421时发现, 其用于检测CTC的C_t为7.075; 而且, 还发现晚期胃癌患者miR-421的水平明显高于早期胃癌患者的水平[34]。我们先通过密度梯度法富集胃癌患者血液中的有核细胞(其中包括CTC), 然后采用RT-PCR技术检测miR-21的水平, 发现CTC关联性miR-21在III/IV期胃癌患者中的水平明显高于I/II期患者的水平; 与健康组比较, 前者平均为(30.46±19.09)倍, 而后者平均只有(19.37±13.67)倍。同时, 我们还证明标本反复冻融不会明显影响CTC关联性miRNA的检测效果[35]。因此, CTC关联性miRNA具有诊断学意义, 以它们作为标记物可用于监测和判断实体瘤患者的临床状态。

3.2 CTC关联性miRNA与肿瘤的治疗

目前常用的抗肿瘤化疗方法具有组织特异性较差、易产生抗药性和毒副作用较大等缺点[36], 而针对肿瘤特异性生物靶标来设计抗癌药物将有可能选择性地治疗肿瘤, 这无疑将为癌症的治疗带来曙光。

Mostert等[29]认为, CTC关联性miRNA在肿瘤的诊断、治疗和预后评估方面均有较大的潜力。研究表明, miRNA对于肿瘤的治疗具有重要的临床意义[37]。Hwang等[38]研究发现, 胰管腺癌(pancreatic ductal adenocarcinoma, PDAC)患者的化疗效果与其miR-21的表达水平呈负相关; 在体外实验中, 反义miR-21可提高抗癌药物的疗效。提示miR-21可能作为一种药物治疗的新型靶点应用于PDAC的治疗。Stenvang等[39]研究发现, 在小鼠和非人类灵长目动物体内, 用修饰的高亲和性寡核苷酸——锁核苷酸(locked nucleic acid, LNA)可介导miR-122的沉默, 预示着基于miRNA的基因治疗方案的可行性。

CTC关联性miRNA有希望成为实体瘤治疗的靶点, 从而为阻止肿瘤转移创造有利条件; 而且基于miRNA的CTC检测可为相关治疗提供疗效评估和筛选个性化治疗方案的依据, 这将有利于改善肿瘤患者的治疗效果。

3.3 CTC关联性miRNA与肿瘤的预后

在先前的研究中, 我们发现CTC关联性miR-21在术前胃癌患者血样中的水平明显高于术后患者, 晚期胃癌患者miR-21的水平明显高于早期胃癌患者, 而后者预后较佳[35]。这些结果提示, CTC关联性miR-21与胃癌的预后呈一定的负相关。同样, CTC关联性miR-106a和miR-17的相关分析也证实了一些致癌性miRNA的水平可预示肿瘤患者的预后[33]。

如上所述, 尽管已有不少研究证明miRNA与肿瘤患者预后的关联性[27,32], 但由于活体肿瘤组织提取所造成的创伤性较大, 病人接受度较低。令人欣慰的是, CTC计数法逐渐发展, 其中前文所述的细胞分析系统已通过美国FDA批准, 可应用于评估转移性乳腺癌、结肠癌和前列腺癌的预后[10,13,40]。基于miRNA的CTC检测更是有着不可否认的高特异性优势, 其发展前景十分可观。CTC的数量已被证明与肿瘤患者OS缩短相关, 应用于临床试验, 提高了病人预后判断的准确性[16]。Poveda等[17]在研究晚期复发性卵巢癌的PFS和OS时发现, CTC数量的增多预示着卵巢癌的不良预后。Mostert等[20]认为, CTC关联性miRNA的价值将随着CTC纯化分离技术和检测手段的进步而日益呈现, 它们将在确定肿瘤原发灶、判断患者预后等方面发挥更大的作用。

4 小结与展望

随着miRNA检测技术的日益成熟, 将miRNA应用于肿瘤的诊断、靶向治疗和预后监测等引起了人

们的广泛兴趣。虽然其中仍有一些未知因素期待解释, 但CTC相关性miRNA的高特异性将有可能为肿瘤的诊断和治疗带来新的希望。基于微创、便捷、高效的血液检测更是为人们所青睐, 我们共同期待有更多令人欣喜的细胞分析技术新进展造福于人类健康。

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Detection of Circulating Tumor Cells Using MicroRNA as A Novel Marker and Its Clinical Significance

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Abstract Circulating tumor cells (CTCs) are solid tumor cells that circulate with blood stream. They are closely associated with the cancer, progress, metastasis, relapse and prognosis. However, the limited number of CTCs makes their efficient detection a big challenge. MicroRNAs (miRNAs), the newly found gene expression regulators, play important roles in cancer recurrence, progress and outcome. The studies on the CTC-associated miRNAs open us mind about CTC detection, and cancer diagnosis and treatment. In this paper, we first introduced the clinical significance of CTCs and the main detection methods, and then summarized the recent research progresses of the relationships between CTC-associated miRNAs, which have been considered as novel tumor cell markers, and cancer diagnosis, treatment and outcome.

Key words microRNA; circulating tumor cells; tumor marker; cell analysis

Received: April 19, 2012 Accepted: June 18, 2012

This work was supported by the National Natural Science Foundation of China (No.81171660), Zhejiang Provincial Research Project (No.2010C33112), Ningbo Natural Science Foundation (No.2010A610044), College Students' Science-Technology Innovation Program of Zhejiang Province (No.2010R405059), the Scientific Innovation Team Project of Ningbo (No.2011B82014), the Project of Key Disciplines in Ningbo (No. XKL11D2127, No.XKL11D2128) and the Research and Development Projects of Experimental Techniques in Ningbo University (No.SYJS-201103)

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