·临床研究·

以癫痫首发的颅内海绵状血管瘤患儿内皮细胞来源外泌体细胞间黏附分子-1表达研究

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【摘要】 **目的** 探讨外泌体细胞间黏附分子-1(ICAM-1)在颅内海绵状血管瘤(CCM)相关性癫痫患者的表达变化及其机制。**方法** 收集2014年2月至2019年2月确诊的儿童癫痫患者临床资料,依据有无合并CCM分为癫痫组和CCM组,并以同期体格检查的健康儿童为对照,每组各20例。通过磁珠捕获技术分选血清外泌体,流式细胞术检测外泌体表面ICAM-1表达;ELISA检测血清ICAM-1含量;免疫组化检测组织内ICAM-1表达变化和$CD68^+$巨噬细胞数目。**结果** 对照组、癫痫组和CCM组受试者血清ICAM-1含量差异无统计学意义($P>0.05$);对照组与癫痫组受试者血清$CD31^+$外泌体ICAM-1平均荧光强度差异无统计学意义($P>0.05$),但CCM组患儿血清$CD31^+$外泌体ICAM-1平均荧光强度较癫痫组增加85.60%[(24.77±3.90)%对(45.97±5.06)%;$t=3.317$,$P=0.008$];海绵状血管瘤组织中ICAM-1平均光密度与巨噬细胞数目呈正相关($r_s=0.909$,$P=0.001$);与对照组相比,共培养组外泌体表面ICAM-1平均荧光强度增加25.61%[(164.81±7.00)%对(207.03±9.18)%;$t=3.652$,$P=0.004$],脂多糖组则进一步增加,相对于共培养组增加71.13%[(354.31±18.22)%对(207.03±9.18)%;$t=7.212$,$P=0.000$],采用白细胞介素-6($t=4.570$,$P=0.001$)和肿瘤坏死因子-α($t=5.105$,$P=0.000$)阻断后外泌体ICAM-1表达水平部分降低。**结论** $CD31^+$外泌体ICAM-1在CCM相关性癫痫患儿体内呈高表达,巨噬细胞与内皮细胞来源外泌体ICAM-1表达变化具有较大的相关性。

【关键词】 血管瘤,海绵状,中枢神经系统; 癫痫; 内皮细胞; 外泌体; 细胞黏附分子; 儿童

Clinical study of intercellular adhesion molecule-1 expression in endothelial-derived exosome in children with intracranial cavernous hemangioma with epilepsy

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【**Abstract**】 **Objective** To investigate the expression and mechanism of exosomal intercellular adhesion molecule-1 (ICAM-1) in patients with intracranial cavernous hemangioma (CCM) associated epilepsy. **Methods** Clinical data of children with epilepsy diagnosed from February 2014 to February 2019 were collected and divided into epilepsy group and CCM group according to the presence or absence of CCM. Healthy children who underwent physical examination during the same period were used as the control group. There were 20 cases in each group. The secretions in serum were sorted by magnetic beads, and ICAM-1 expression was detected by flow cytometry. Serum ICAM-1 was detected by enzyme-linked immunsorbent assay (ELISA). The expression of ICAM-1 and the number of $CD68^+$ macrophages were detected by immunohistochemistry. **Results** The results of ELISA showed that there was no significant difference in serum ICAM-1 concentration among the control group, epilepsy group and CCM group ($P>0.05$). There was no significant difference in the average fluorescence intensity of $CD31^+$ exosome ICAM-1 between the control group and the epilepsy group. Compared with the epilepsy group, the average fluorescence intensity of $CD31^+$ exosome ICAM-1 in the CCM group increased by 85.60% [(24.77±3.90)% vs. (45.97±5.06)%; $t=3.317$, $P=0.008$]. The average optical density of ICAM-1 in CCM tissue was positively correlated with the number of macrophages ($r_s=0.909$, $P=0.001$). The results of co-culture

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experiment showed that compared with the control group, the average fluorescence intensity of ICAM-1 on the exosome surface of co-culture group increased by 25.61% [(164.81 ± 7.00)% vs. (207.03 ± 9.18)%; t = 3.652, P = 0.004]; the average fluorescence intensity of ICAM-1 on the exosome surface of LPS group further increased by 71.13% [(354.31 ± 18.22)% vs. (207.03 ± 9.18)%; t = 7.212, P = 0.000]; the blockade of IL-6 (t = 4.570, P = 0.001) and TNF-α (t = 5.105, P = 0.000) could partly reduce the expression of ICAM-1 on the exosome. **Conclusions** The expression of $CD31^+$ exosome ICAM-1 is significantly increased in patients with CCM associated epilepsy. There is a strong correlation between the expression of macrophages and endothelial cell-derived exosome ICAM-1.

【Key words】 Hemangioma, cavernous, central nervous system; Epilepsy; Endothelial cells; Exosomes; Cell adhesion molecules; Child

Conflicts of interest: none declared

颅内海绵状血管瘤(CCM)是一种先天性颅内血管畸形病变,约50%患者以癫痫为首发症状[1]。该病可发生于任何年龄,但儿童患者尤其是以癫痫为首发症状的患儿常因表达不完全或不能配合相关检查而被误诊,因而不能得到及时有效的治疗,导致严重后果[2-3]。因此,寻找新的具有快速诊断意义的生物学标志物具有重要临床意义。细胞间黏附分子-1(ICAM-1)属于黏附分子免疫球蛋白超家族,可介导炎性细胞与血管内皮细胞间的粘附,在冠心病和脑出血等疾病的监测中具有潜在的临床应用前景[4-5],其血清水平变化可作为诊断和监测海绵状血管瘤疗效的生物学标志物。然而,血清ICAM-1变化极易受微环境等外界因素的影响,导致实验室检测呈假阴性或假阳性[6]。外泌体是由细胞分泌的膜性囊泡样物质,可在血清中长时间存在,其表面蛋白质表达稳定,分泌后极少受到外界环境因素的影响,故在疾病诊断和治疗过程中具有重要作用[7-9]。研究表明,于磁珠表面包被CD63抗体,可特异性捕获血液循环中的外泌体,通过流式细胞术检测外泌体表面蛋白质的表达变化即可准确而迅速地确定血清ICAM-1含量,而且价格低廉,目前已有相关产品进入临床试验,应用于疾病的快速诊断和监测[10],本文拟对外泌体ICAM-1在颅内海绵状血管瘤致儿童癫痫诊断中的作用进行观察,并探讨其发生机制。

对象与方法

一、观察对象

1.纳入与排除标准 (1)纳入标准:临床症状与脑电图检查均符合2001年国际抗癫痫联盟(ILAE)癫痫分类中有关原发性癫痫的诊断标准[3];MRI显示网状混杂信号,周围呈明显T_2WI低信号圈;年龄>18岁;患儿家属对检查项目及相关风险知情并签署知情同意书。(2)排除标准:合并以下疾病者均非本研究观察对象,包括恶性肿瘤、获得性免疫缺陷综合征(AIDS)或乙型病毒性肝炎等感染性疾病、先天性心脏病等遗传性疾病,以及冠心病或血管炎等其他心血管疾病。

2.一般资料 根据上述纳入与排除标准,选择2014年2月至2019年2月在南通大学附属医院儿科就诊的癫痫患儿共40例,根据其临床表现和影像学是否合并颅内海绵状血管瘤,分为单纯癫痫发作组(癫痫组)和海绵状血管瘤相关性癫痫组(CCM组),每组各20例。两组患儿均以癫痫为首发症状,脑电图表现为散在或连续出现的棘波节律或双侧对称性同步棘-慢综合脑电异常信号,癫痫组部分性发作6例、全面性强直-阵挛发作14例,CCM组部分性发作4例、全面性强直-阵挛发作16例。CCM组患儿T_1WI呈低信号、T_2WI呈高信号,在控制癫痫发作后均行单纯海绵状血管瘤切除手术。选择20例同期在我院进行体格检查的健康儿童作为正常对照(对照组),对三组受试者性别、年龄、体重参数进行比较差异无统计学意义(均P>0.05,表1),均衡可比。

二、研究方法

1.实验材料 (1)血液标本:采集各组受试者手部静脉血1 ml,分离血清并置于-80℃冰箱保存,用于血清外泌体ICAM-1的检测。(2)试剂与药品:人内皮细胞系(EC-304,3~5代)和巨噬细胞系(U937,3~5代)由美国American Type Culture Collection公司提供。外泌体-人CD63分离/检测试剂盒购于美国Invitrogen公司。小鼠抗人CD63-PE、CD31-APC和ICAM-1-FITC单克隆抗体为美国BD公司产品,ICAM-1酶联免疫吸附试验(ELISA)检测试剂盒由深圳欣博盛生物科技有限公司提供。无外泌体血

表1 三组受试者社会人口学资料的比较

Table 1. Comparison of sociodemographic data of the subjects in 3 groups

组别	例数	年龄（$\bar{x}\pm s$，岁）	性别[例(%)]		体重（$\bar{x}\pm s$，kg）
			男性	女性	
对照组	20	7.16±1.25	12(12/20)	8(8/20)	24.38±1.68
癫痫组	20	5.93±0.61	14(14/20)	6(6/20)	20.17±1.54
CCM组	20	6.03±0.90	12(12/20)	8(8/20)	19.50±1.29
F或χ^2值		0.514	0.574		2.917
P值		0.608	0.751		0.085

χ^2 test for comparison of sex and ANOVA test for comparison of the others，性别的比较采用χ^2检验，其余项目的比较采用单因素方差分析。CCM，intracranial cavernous hemangioma，颅内海绵状血管瘤

清、胎牛血清、RPMI 1640培养基，以及脂多糖（LPS）分别购于美国Hyclone或Sigma公司。Transwell细胞共培养小室购于美国Corning公司。（3）仪器与设备：LSRFortessa X-20流式细胞仪由美国BD公司提供，TG16KR高速冷冻离心机购自上海继谱电子科技有限公司。

2. 检测方法 （1）流式细胞术检测血清外泌体表达变化：将三组受试者外周血分别置15 ml离心管，离心半径10 cm、1500 r/min高速离心10 min，取上清液，滴加5 μl免疫磁珠CD63，室温孵育4 h；置磁力架上1 min以结合血清中的外泌体，弃上清液。上述实验步骤共重复3次，然后分别滴加CD63（2 μl）、CD31（5 μl）和ICAM-1（5 μl）流式细胞仪检测相应抗体，室温避光孵育1 h，置磁力架上1 min，弃上清液。重复3次后滴加150 μl磷酸盐缓冲液（PBS）行流式细胞术检测$CD31^+$外泌体表面ICAM-1表达水平，$CD63^+$代表免疫磁珠所捕获的为外泌体。（2）ELISA试验检测血清ICAM-1表达变化：采用ELISA试剂盒检测三组患儿血清ICAM-1水平，实验过程严格按照试剂盒说明书操作。（3）组织病理观察：采集CCM组患儿手术切除标本，以质量分数为10%的甲醛溶液固定、石蜡包埋，制备厚度为3 μm的海绵状血管瘤组织切片，每一标本共制备3张脑组织切片，分别行HE染色、ICAM-1和CD68免疫组化染色，检测海绵状血管瘤组织ICAM-1表达和$CD68^+$巨噬细胞浸润情况；以染色呈紫色或黑色者为免疫组化表达阳性。（4）细胞培养：内皮细胞和巨噬细胞分别置于含体积分数为10%胎牛血清的RPMI 1640培养基中进行培养，并于巨噬细胞培养基中加入10 ng/ml LPS以模拟炎症环境，刺激48 h后收集巨噬细胞进行后续共培养实验。（5）细胞共培养：单细胞培养4～6 d，于Transwell共培养小室内对巨噬细胞和内皮细胞共培养48 h。然后，采集呈对数生长期的内皮细胞（20×10^3），以质量分数为0.25%胰蛋白酶1 ml消化2 min，平均传代至24孔板下室，细胞贴壁后PBS冲洗（×3）并分为对照组（加入相同体积PBS）、共培养组（Transwell上室加入巨噬细胞）、LPS组（Transwell上室加入经LPS预刺激巨噬细胞）、白细胞介素-6（IL-6）阻断组（Transwell上室加入经LPS预刺激巨噬细胞＋IL-6中和性抗体），以及肿瘤坏死因子α（TNF-α）阻断组（Transwell上室加入LPS预刺激巨噬细胞＋TNF-α中和性抗体）。培养48 h后去上室细胞、PBS连续冲洗（×3），滴加体积分数10%不含外泌体血清的RMPI 1640培养基中继续培养48 h，收集上清液，流式细胞术检测外泌体表面ICAM-1表达的平均荧光强度。

3. 统计分析方法 采用SPSS 17.0统计软件进行数据处理与分析。计量资料以均数±标准差（$\bar{x}\pm s$）表示，多组间的比较采用单因素方差分析，两两比较行Bonferroni法；计数资料以相对数构成比（%）或率（%）表示，采用χ^2检验；相关性分析行Spearman秩相关。以$P\leqslant0.05$为差异具有统计学意义。

结 果

ELISA检测显示，各组受试者血清ICAM-1表达水平差异无统计学意义（$F=0.751$，$P=0.489$；表2）。流式细胞术检测，经磁珠分离获得的外泌体95%以上表达CD63，表明外泌体纯度较高，可用于后续实验，进一步检测结果显示，对照组、癫痫组和CCM组患儿血清$CD31^+$外泌体比例差异无统计学意义（$F=0.356$，$P=0.708$）；对照组与癫痫组患儿$CD31^+$外泌体ICAM-1平均荧光强度差异无统计学意义（$t=1.016$，$P=0.334$）；与癫痫组相比，CCM组患儿$CD31^+$外泌体ICAM-1平均荧光强度增加85.60%［（24.77±3.90）%对（45.97±5.06）%；$t=3.317$，$P=0.008$；表3，4］。

免疫组化染色显示，海绵状血管瘤组织中内皮细胞表面ICAM-1表达呈强阳性，$CD68^+$巨噬细胞数目为11.20个/视野，且ICAM-1平均光密度与巨噬细胞数目呈正相关（$r_s=0.909$，$P=0.001$），提示巨噬细胞可能是引起海绵状血管瘤内皮细胞表面ICAM-1

表2　三组受试者血清ICAM-1表达水平的比较($\bar{x} \pm s$, ng/ml)

Table 2. Comparison of serum ICAM-1 levels in 3 groups ($\bar{x} \pm s$, ng/ml)

组别	例数	ICAM-1
对照组	20	14.55 ± 1.56
癫痫组	20	16.80 ± 1.87
CCM组	20	20.72 ± 2.21
*F*值		0.751
*P*值		0.489

CCM, intracranial cavernous hemangioma, 颅内海绵状血管瘤；ICAM-1, intercellular adhesion molecule-1, 细胞间黏附分子-1。The same for Table 3

表3　三组受试者血清CD31$^+$外泌体ICAM-1表达变化的比较($\bar{x} \pm s$, %)

Table 3. Comparison of changes in serum CD31$^+$ exosome ICAM-1 expression among subjects in 3 groups ($\bar{x} \pm s$, %)

组别	例数	CD31$^+$-ICAM-1
对照组(1)	20	19.80 ± 2.92
癫痫组(2)	20	24.77 ± 3.90
CCM组(3)	20	45.97 ± 5.06
*F*值		11.731
*P*值		0.001

表4　三组受试者血清CD31$^+$外泌体ICAM-1表达变化的两两比较

Table 4. Pairwise comparison of changes in serum CD31$^+$ exosome ICAM-1 expression among subjects in 3 groups

组间两两比	CD31$^+$-ICAM-1	
	*t*值	*P*值
(1)与(2)	1.016	0.334
(1)与(3)	4.476	0.001
(2)与(3)	3.317	0.008

ICAM-1, intercellular adhesion molecule-1, 细胞间黏附分子-1

表5　各组受试者巨噬细胞促进内皮细胞来源的外泌体ICAM-1表达水平的比较($\bar{x} \pm s$, %)

Table 5. Comparison of the expression levels of ICAM-1 in exosomes promoted by macrophages in different groups ($\bar{x} \pm s$, %)

组别	例数	ICAM-1
对照组(1)	6	164.81 ± 7.00
共培养组(2)	6	207.03 ± 9.18
LPS组(3)	6	354.31 ± 18.22
IL-6阻断组(4)	6	256.06 ± 11.38
TNF-α阻断组(5)	6	248.11 ± 10.02
*F*值		35.802
*P*值		0.000

LPS, lipopolysaccharides, 脂多糖；IL-6, interleukin-6, 白细胞介素-6；TNF-α, tumor necrosis factor-α, 肿瘤坏死因子-α；ICAM-1, intercellular adhesion molecule-1, 细胞间黏附分子-1

表6　不同组别巨噬细胞促进内皮细胞来源的外泌体ICAM-1表达变化的两两比较

Table 6. Pairwise comparison of the changes of ICAM-1 expression in exosomes promoted by macrophages in different groups

组间两两比	ICAM-1		组间两两比	ICAM-1	
	*t*值	*P*值		*t*值	*P*值
(1)与(2)	3.652	0.004	(2)与(4)	3.351	0.007
(1)与(3)	9.700	0.000	(2)与(5)	3.016	0.013
(1)与(4)	6.825	0.000	(3)与(4)	4.570	0.001
(1)与(5)	6.804	0.000	(3)与(5)	5.105	0.000
(2)与(3)	7.212	0.000	(4)与(5)	0.528	0.609

ICAM-1, intercellular adhesion molecule-1, 细胞间黏附分子-1

表达增强的主要机制。

与对照组相比，共培养组外泌体表面ICAM-1平均荧光强度增加25.61%[(164.81 ± 7.00)%对(207.03 ± 9.18)%；$t = 3.652$, $P = 0.004$]，而LPS组外泌体表面ICAM-1平均荧光强度则进一步增加，相对于共培养组增加71.13%[(354.31 ± 18.22)%对(207.03 ± 9.18)%；$t = 7.212$, $P = 0.000$]；经IL-6和TNF-α阻断后，可使部分外泌体ICAM-1表达水平下降，与LPS组相比，分别降低27.72%[(256.06 ± 11.38)%对(354.31 ± 18.22)%；$t = 4.570$, $P = 0.001$]和29.97%[(248.11 ± 10.02)%对(354.31 ± 18.22)%；$t = 5.105$, $P = 0.000$；表5,6]。

讨　论

癫痫是由大脑神经元异常放电引起的短暂性大脑功能障碍性病变，病因十分复杂，颅内海绵状血管瘤特别是海绵状血管瘤出血诱发癫痫发作的患儿，需迅速处理并针对病因进行治疗[11]。然而，大多数患儿由于疼痛而拒绝配合检查，造成癫痫症状掩盖对原发病的发现或诊断，从而延误治疗并预后不良。对本研究所纳入患儿的观察结果显示，海绵状血管瘤相关性癫痫组患儿血清ICAM-1表达水平与单纯癫痫发作组之间差异无统计学意义。大量研究业已证实，外周血细胞因子的表达极易受到外界环境的干扰，而且一些非海绵状血管瘤组织细胞亦可以分泌ICAM-1，在情绪激动或者疾病状态下即可诱导ICAM-1释放，使其在外周血中的含量显著升高[12-13]；而外泌体ICAM-1的表达变化相对于血清ICAM-1更稳定、准确性更高，具备作为生物学标志物之特性。

外泌体在细胞信号交流和行使功能的过程中发挥重要作用，被认为是目前最具发展前景的临床检测技术[14-16]，通过检测外泌体蛋白质表面细胞程序性死亡蛋白1（PD1）等分子的表达，可以预测人体内是否发生肿瘤，以及针对肿瘤细胞所实施治疗的效果[17]。由内皮细胞分泌的各种外泌体均具有促进血管生成和调控炎症反应的作用[18]，本研究海绵状血管瘤相关性癫痫组患儿外周血$CD31^+$外泌体蛋白表面ICAM-1表达显著高于单纯癫痫组患儿，相对于单纯外周血细胞因子，外泌体具有更高的稳定性，不易受内环境因素的影响。已知在外泌体表面存在一些特异性较强的分子，其表达稳定，是一种较好的外泌体来源性标志物，本研究采用CD31对内皮细胞来源的外泌体进行标记，显示出良好的可重复性和临床应用价值。

ICAM-1广泛存在于机体组织器官，参与多种疾病的发生与发展过程，发挥免疫调节和免疫应答等重要功能[19-20]，目前ICAM-1更多用于评价心肌梗死、支气管扩张、恶性肿瘤或肾小球肾炎等疾病的严重程度[21]。与上述疾病相比，颅内海绵状血管瘤尤其是隐匿性海绵状血管瘤病变区域狭小、局限，因此根据外周血ICAM-1表达变化预测海绵状血管瘤严重程度的临床价值尚存争议，由于纳入标准不同，使得各项研究的结论也存在较大差异。本研究结果表明，外泌体ICAM-1预测海绵状血管瘤相关性癫痫具有临床筛查和诊断价值；而其表达变化与巨噬细胞比例呈正相关，后者主要通过分泌IL-6和TNF-α等炎性因子而诱导内皮细胞释放ICAM-1表达阳性外泌体，炎症反应越强、外泌体ICAM-1表达水平越高，提示外周血巨噬细胞数目可作为评价ICAM-1表达的指标。

综上所述，$CD31^+$外泌体ICAM-1在颅内海绵状血管瘤相关性癫痫患儿外周血中呈高表达，而巨噬细胞与内皮细胞来源外泌体ICAM-1的表达存在显著相关性，其深入的病理生理学机制尚需进一步研究，但笔者认为外泌体ICAM-1的表达变化，可作为海绵状血管瘤相关性癫痫患儿的新的筛查指标。

利益冲突 无

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Echography and Doppler of the Brain published

Echography and Doppler of the Brain (eBook ISBN 978-3-030-48202-2; Hardcover ISBN 978-3-030-48201-5) will be published by Springer in 2021. The authors of this book are Chiara Robba and Giuseppe Citerio.

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About the book: the aim of this book is to educate and train practitioners in the safe and professional use of diagnostic ultrasound imaging in the visualization and interpretation of various cerebral conditions not only in neurointensive care, but also in the operating room and, in general, cardiothoracic and neurocritical care settings. It is chiefly intended for anaesthetists and intensivists with a basic knowledge of ultrasound physics, but also for neurosurgeons and neurologists. All chapters were coordinated by the Editors, with experiences in hands-on courses on Echography and Doppler of the Brain, and prepared by international experts. The book covers from basic principles to estimation of intracranial pressure and cerebral perfusion. The topics cover emergency department and prehospital brain US as part of POCUS and US multiorgan evaluation to general intensive care, neurointensive care and anesthesia, including special populations as pregnant and children and setting as LMIC. Clinical scenarios complete the book. An innovative and unique guide that equips readers to perform bedside and non-invasive assessments for a range of cerebrovascular diseases. The price of the eBook is 74.89€, and the hardcover is 93.59€. Visit link.springer.com for more information.