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研究论文

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基于转录组测序挖掘仿刺参“化皮病”相关基因

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摘要: 为了寻找仿刺参(*Apostichopus japonicus*)养殖期间的“化皮病”关键调控基因, 并分析这些基因所参与的信号通路, 对本课题组前期已获得的仿刺参“化皮” I 期(早期)、II 期(中期)和 III 期(后期)3 个阶段的病变及其同一个体正常体壁组织之间的差异表达基因(differentially expressed genes, DEGs)进行进一步分析。主成分分析(principal component analysis, PCA)结果显示, “化皮” II 期病变组织与正常组织之间的差异最小, “化皮” I 期与 III 期表达关系比较接近, “化皮” II 期是一个“转折期”。KEGG 富集分析结果显示, 补体与凝血级联(Complement and coagulation cascades)通路和细胞外基质受体(ECM-receptor interaction)通路在“化皮” 3 个阶段都显著改变。通过构建“化皮”过程关键差异表达基因调控网络, 发现 IgGFc-binding protein (FcGBP)基因和 Tenascin(TN)蛋白家族基因在“化皮”不同阶段参与到发生显著变化的信号通路。qRT-PCR 验证结果显示, 5 个 DEGs 在仿刺参“化皮”不同阶段表达趋势与 RNA-Seq 结果一致, 皮尔逊相关系数 *r* 值为 0.7714。“化皮”过程关键调控基因的筛选将为抗逆品种选育以及“化皮病”的防控提供科学依据。

关键词: 仿刺参; 化皮; 差异表达基因; 调控网络
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仿刺参(*Apostichopus japonicus*), 又称刺参, 是中国山东、辽宁等北方地区重要的增养殖品种。随着仿刺参养殖业的快速发展, 其病害问题日趋严重, “腐皮综合症”(俗称“化皮病”)是当前养殖刺参最主要的疾病, 在一些地区已经造成严重的经济损失[1-2]。大量的病原病理学研究证实, 仿刺参“化皮病”主要是以细菌感染为主, 其中灿烂弧菌(*Vibrio splendidus*)是造成该病发生的主要病原[1-3]。温度、盐度、亚硝酸盐含量和硫化物含量等环境因素的变化也与病害发生有关[4-6]。近年来, 有关仿刺参在“化皮”过程中的免疫调控机制研究取得了一定的进展, 研究者已经克隆了多个免疫基因和信号通路中关键调控因子的 cDNA 全长序列, 并分析了这些基因在病原感染后的时空表达特征, 包括溶菌酶[7]、补体 C3、补体 Bf[8-10]、凝集素[11]、酚氧化酶[12]、转录因子 FoxO[13]以及 Toll 样受体信号通路[14-16]和 TNF-α 信号通路[17]中的关键调控因子等。随着分子生物学高新技术及研究手段的迅速发展, 仿刺参“化皮病”相关转录组学[18-23]、蛋白组学[24-26]和代谢组学[27]的研究也相继开展, 为揭示其分子水平上的调控机理奠定了基础。

项目组前期通过灿烂弧菌感染获得了仿刺参“化皮病”实验群体, 并根据体壁组织的“化皮”程度将病变阶段人为地分为 I 期、II 期和 III 期 3 个阶段, 采用 RNA-Seq 技术对健康和“化皮”仿刺参的不同组织(包括体壁、肠、呼吸树和体腔细胞)、“化皮”不同阶段的病变及其同一个体正常体壁组织进行了转录组测序[23]。本研究在此基础上, 对

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已获得的仿刺参“化皮”不同阶段的病变和正常体壁组织间的差异表达基因进行了主成分分析(principal component analysis, PCA)、韦恩(Venn)分析及 KEGG(Kyoto Encyclopedia of Genes and Genomes)富集分析; 通过构建“化皮”过程差异表达基因调控网络, 挖掘与仿刺参“化皮”相关的基因。研究结果将为仿刺参“化皮”过程关键调控基因的筛选以及“化皮病”的防控提供科学依据。

1 材料与方法

1.1 数据来源及分析

仿刺参“化皮病”试验群体的构建、转录组测序及差异表达基因的筛选来源于 Yang 等[23]。即通过灿烂弧菌感染, 构建仿刺参“化皮病”试验群体, 分成 I 期(早期)、II 期(中期)和 III 期(后期)3 个病变阶段。“化皮”I 期: 仿刺参体壁出现一个白色溃疡斑点(直径<0.2 cm), 未吐脏, 具有附着能力; “化皮”II 期: 仿刺参体壁出现 2~3 个溃疡斑点(直径 0.2~0.5 cm), 未吐脏, 具有附着能力; “化皮”III 期: 仿刺参体壁出现深度大面积溃疡, 吐脏, 失去附着能力。基于 Illumina HiSeq 2000 测序平台, 对健康群体的体壁、肠、呼吸树、体腔细胞、“化皮” 3 个阶段同一个体的病变体壁组织和正常体壁组织以及“化皮”中期的肠、呼吸树、体腔细胞进行了转录组测序。以差异倍数≥2 且错误发现率(false discovery rate, FDR) < 0.001 为标准, 进行了不同发病阶段、不同组织间及同一“化皮”个体病变组织与正常组织之间差异表达基因的分析。差异倍数表示两组样品间基因表达量(RPKM 值)的比值。错误发现率是通过使用 Benjamini-Hochberg 校正方法对差异显著性 *P* 值(*P*-value)进行校正得到的[28]。本研究对已获得的“化皮” 3 个阶段的病变的组织及其同一个体正常体壁组织之间的 646、127 和 568 个差异表达基因使用 R 软件(R-2.15.3)进行 PCA 分析和韦恩图的绘制。

1.2 差异表达基因的 KEGG 富集分析

Pathway 显著性富集分析以 KEGG Pathway 为单位, 应用超几何检验, 找出与整个转录组背景相比, 在差异表达基因中显著性富集的 Pathway。该假设检验的 *P*-value 计算公式为:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

式中, *N* 为所有 Unigene 中具有 Pathway 注释的基因数目, *n* 为 *N* 中差异表达基因数目, *M* 为所有 Unigene 中注释为某特定 Pathway 的基因数目, *m* 为被注释成某特定 Pathway 的差异表达基因数目。经过多重检验校正后, 选择 Q value≤0.05 的 Pathway 定义为在差异表达基因中显著富集的 Pathway。

1.3 仿刺参“化皮”过程关键差异表达基因调控网络构建

利用 KEGG 富集分析筛选仿刺参“化皮” 3 个阶段均发生显著变化的通路, 将通路中的相关基因列表导入 Cytoscape3.2.1 软件, 构建仿刺参“化皮”过程关键差异表达基因的调控网络图。

1.4 差异表达基因的 qRT-PCR 验证

选取 Fibrinogen C domain-containing protein 1-like(*FIBCD1*)、IgGFc-binding protein(*FcGBP*)、Complement H(*CFH*)、Metalloproteinase inhibitor 1-like(*TIMP1*)和 GalNAc-specific lectin(*GalNAc*)基因进行 qRT-PCR 验证。采用 Yang 等[23]的方法再次通过灿烂弧菌(*V. splendidus*)感染获得仿刺参“化皮病”I 期、II 期和 III 期实验群体并提取“化皮”不同阶段的病变及其同一个体正常体壁组织的 RNA。每个样品的总 RNA 用 PrimeScript™ RT reagent Kit(TaKaRa)进行反转录, 反应体积及反应条件按照说明书进行。利用 Primer 5.0 软件, 根据转录组获得的基因序列信息, 设计 qRT-PCR 实验所需引物, 内参采用 Cyt *b* 基因[29]。引物序列信息见表 1。qRT-PCR 采用 SYBR Green I 染料法(SYBR PrimeScript™ RT-PCR Kit II, TaKaRa), 在 Mx3005p™ 荧光定量 PCR 仪(Stratagene, La Jolla, CA, USA)上进行。反应终体积 20 μL, 包括 10 μL 2×SYBR Premix Ex Taq™ II (*Tli* RnaseH Plus), 0.4 μL ROX Reference Dye II, 2 μL cDNA 样品, 引物各 0.4 μmol/L。反应条件: 95℃, 30 s; 95℃ 10 s,

表 1　qRT-PCR 验证引物列表
Tab. 1　Primers used for qRT-PCR validation

基因名称 gene	引物序列(5′–3′) primer sequence (5′–3′)
细胞色素 b cytochrome b	Cytb-F: TGAGCCGCAACAGTAATC Cytb-R: AAGGGAAAAGGAAGTGAAAG
纤维蛋白原 C 结构域蛋白 1 样蛋白 fibrinogen C domain-containing protein 1-like	FIBCD1-F:CCAAAGGAACACTACGATGGA FIBCD1-R:AGTAAGACGCCAGTTGAGACG
IgGFc 片段结合蛋白 IgGFc-binding protein	FcGBP-F: TCCCGTTCCCATAAATCTCA FcGBP-R: GCTGCGAGATCCTTTTGTTC
补体 H complement H	CFH-F: TGGTTCTGTGTCCAATGTCC CFH-R: GGTGAAATGGTAGTGCGAAAC
金属蛋白酶抑制剂 1 样蛋白 metalloproteinase inhibitor 1-like	TIMP1-F: AAGTTGCCTCCCGAATGTT TIMP1-R: TGAATGAAGACTGCGAAACC
N-乙酰半乳糖胺特异性凝集素 GalNAc-specific lectin	GalNAc-F:CCATCCTTCAGGGCAGATAA GalNAc-R:TTCATCGACCAAAATGCAGA

56℃ 25 s, 72℃ 25 s, 40 个循环。采用 $2^{-\Delta\Delta Ct}$ 法计算 mRNA 相对表达量[30]。采用皮尔逊相关系数(Pearson correlation coefficient)分析 RNA-Seq 和 qRT-PCR 结果的相关程度, 皮尔逊相关系数 r 取值范围在[–1, 1] [31]。

2　结果与分析

2.1　仿刺参“化皮”不同阶段的病变和正常体壁组织间差异表达基因分析

前期研究已经对仿刺参“化皮” 3 个阶段的病变及其同一个体正常体壁组织进行了差异表达基因的分析, “化皮” I 期、II 期和 III 期的差异表达基因分别为 646、127 和 568 个[23]。本研究在此基础上, 进行了 PCA 分析, 结果显示, “化皮” II 期病变组织与正常组织之间的差异最小; “化皮” I 期与 III 期表达关系比较接近, “化皮” II 期是一个“转折期” (图 1)。对 3 组差异基因(646、127 和 568 个)进行韦恩图绘制, 结果显示, 3 个阶段特异表达的基因分别为 497、59 和 433 个, 分别占差异表达基因总数的 76.93%、46.46%和 76.23%; 3 个阶段共同表达的基因仅为 28 个(图 2)。仿刺参“化皮” 3 个阶段的病变及其同一个体正常体壁组织间前 10 个最显著并获得注释的差异表达基因(top 10DEGs)见表 2。其中, 只有 *FcGBP* 基因在“化皮” I 期下调表达, 而在“化皮” III 期上调表达, 其余 DEGs 均为阶段性特异表达基因。

2.2　差异表达基因的 KEGG 富集分析

通过 Pathway 显著性富集能确定差异表达基因参与的主要生化代谢途径和信号转导途径。分

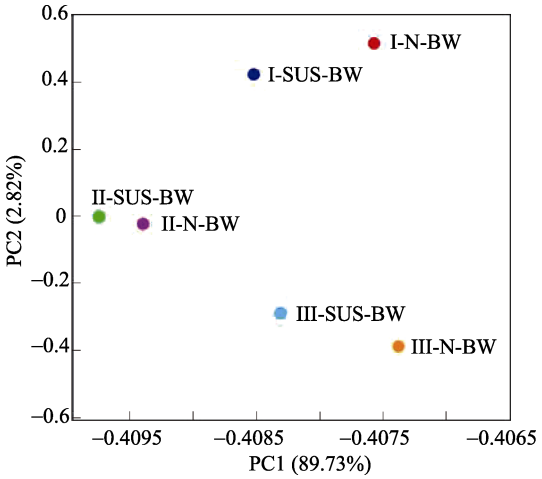

图 1　仿刺参“化皮”不同阶段病变和正常体壁组织间差异表达基因的 PCA 分析
BW: 体壁组织; SUS: “化皮”组织; N:正常组织; I: “化皮” I 阶段; II: “化皮” II 阶段; III: “化皮” III 阶段.
Fig. 1　PCA of DEGs between SUS and normal body walls from three stages of SUS progression
BW: body wall; SUS: skin ulceration syndrome; N: normal tissue; I: SUS stage I; II: SUS stage II; III: SUS stage III.

别对 3 组差异基因进行 KEGG 富集分析(Q value≤0.05), “化皮” 3 个阶段上调基因分别参与了 2、1、6 条显著性信号通路; 下调基因分别参与了 3、4、1 条显著性信号通路(表 3)。补体与凝血级联(complement and coagulation cascades)通路和细胞外基质受体相互作用(ECM-receptor interaction)通路在“化皮” 3 个阶段都显著改变, 其中细胞外基质受体通路在“化皮” I 和 II 期发生了下调改变, 但是在“化皮” III 期却发生上调改变。细胞黏附(focal adhesion)通路在“化皮” II 阶段发生了下调改变, 但是在“化皮” III 期发生了上调改变。补体

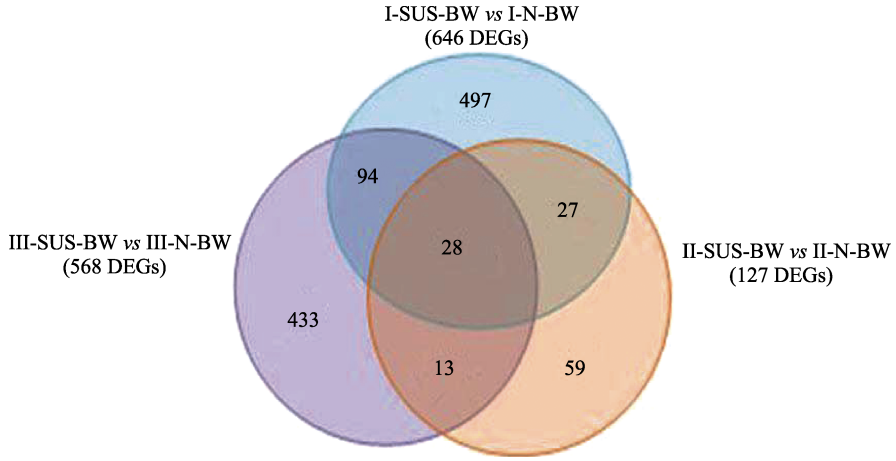

图 2 仿刺参“化皮”不同阶段差异基因的韦恩图分析

BW: 体壁组织; SUS: “化皮”组织; N:正常组织; I: “化皮” I 阶段; II: “化皮” II 阶段; III: “化皮” III 阶段.

Fig. 2 The distributions of DEGs from three stages of SUS progression through Venn diagram

BW: body wall; SUS: skin ulceration syndrome; N: normal tissue; I: SUS stage I; II: SUS stage II; III: SUS stage III.

表 2 仿刺参“化皮”不同阶段的病变和正常体壁组织间前 10 个最显著的差异表达基因

Tab. 2 Top 10 annotated DEGs between SUS and normal body walls during skin ulceration syndrome (SUS) progression

排序 order	基因注释 gene annotation	$\log_2$(化皮/正常) $\log_2$(SUS/N)	FDR 值 FDR value
	“化皮” I 阶段 SUS stage I		
1↑	胶蛋白 cement protein	9.1786	8.69E-05
2↑	氨肽酶 N 异构体 1 aminopeptidase N isoform 1	7.5000	2.26E-16
3↑	纤维蛋白原 C 结构域蛋白 1 样蛋白 fibrinogen C domain-containing protein 1-like	4.7677	2.83E-06
4↑	视黄醇脱氢酶 8 样蛋白 retinol dehydrogenase 8-like	4.2184	4.15E-08
5↑	天冬氨酸转氨酶, 线粒体样亚型 2 aspartate aminotransferase, mitochondrial-like isoform 2	3.8911	0
6↑	ras 相关肉毒杆菌毒素底物 1 ras-related C3 botulinum toxin substrate 1	3.5612	1.67E-04
7↑	钠依赖性磷酸转运蛋白 2B 样蛋白 sodium-dependent phosphate transport protein 2B-like	2.9983	1.30E-06
8↑	唾液腺分泌蛋白 1 salivary gland secretion 1	2.6909	1.12E-08
9↑	金属蛋白酶抑制剂 3 样蛋白 metalloproteinase inhibitor 3-like	2.6735	0
10↑	丙二烯氧化物合成酶-脂氧合酶样蛋白 allene oxide synthase-lipoxygenase protein-like	2.6586	0
1↓	过氧化物酶样蛋白 peroxidase-like	–5.6310	0
2↓	乳过氧化物酶 lactoperoxidase	–5.4611	7.41E-66
3↓	表皮生长因子 epidermal growth factor	–5.2181	0
4↓	神经源性轨迹切口同族蛋白 2 样蛋白 neurogenic locus notch homolog protein 2-like	–4.5986	3.68E-05
5↓	Hedgling 样蛋白 hedgling-like	–4.3091	4.61E-04
6↓	胰腺转录因子 1 亚单位 α 样蛋白 pancreas transcription factor 1 subunit alpha-like	–4.3091	4.61E-04
7↓	妊娠区带蛋白样蛋白 pregnancy zone protein-like	–4.2387	3.18E-28
8↓	GLI 的发病机制相关的 1(胶质瘤)样基因 GLI pathogenesis-related 1 (glioma)-like	–3.9627	0
9↓	7B tetratricopeptide 重复蛋白亚型 2 tetratricopeptide repeat protein 7B isoform 2	–3.6417	6.59E-07
10↓	IgGFc 片段结合蛋白 IgGFc-binding protein	–2.6164	1.34E-39

(待续 to be continued)

(续表 2 Tab. 2 continued)

排序 order	基因注释 gene annotation	log_2(化皮/正常) log_2(SUS/N)	FDR 值 FDR value
	“化皮” II 阶段 SUS stage II		
1↑	前蛋白转化酶枯草溶菌素 9 proprotein convertase subtilisin/kexin type 9	4.5389	2.43E-21
2↑	微管蛋白 α 亚基 tubulin alpha subunit	3.5166	1.66E-10
3↑	血小板衍生生长因子受体 α 样蛋白 platelet-derived growth factor receptor alpha-like	1.9454	1.63E-04
4↑	线粒体 mid 同源蛋白样蛋白 protein midA homolog, mitochondrial-like	1.4724	5.32E-05
5↑	金属蛋白酶抑制剂 1 样蛋白 metalloproteinase inhibitor 1-like	1.1112	9.10E-20
1↓	细胞色素 c 氧化酶亚单位 I(线粒体) cytochrome c oxidase subunit I (mitochondrion)	–10.1747	9.08E-09
2↓	ATP 合成酶 F0 亚基 6 ATP synthase F0 subunit 6	–9.4991	1.34E-06
3↓	α-肌动蛋白-1 Alpha-actin-1	–9.1606	3.11E-12
4↓	芳香基硫酸酯酶样蛋白 arylsulfatase-like	–4.0359	1.33E-29
5↓	补体 H complement H	–3.0680	4.13E-16
6↓	磷脂酶 A2 AP-PLA2-II 样蛋白 phospholipase A2 AP-PLA2-II-like	–2.4949	1.48E-17
7↓	钠依赖性磷酸转运蛋白 2B 样蛋白 sodium-dependent phosphate transport protein 2B-like	–2.2395	3.51E-04
8↓	Sushi 域蛋白-2 样蛋白 sushi domain-containing protein 2-like	–2.1615	1.82E-11
9↓	Techylectin-5A 样蛋白亚型 X1 techylectin-5A-like isoform X1	–2.1011	1.66E-29
10↓	麦芽糖酶葡糖淀粉酶 maltase-glucoamylase	–2.0910	1.24E-08
	“化皮” III 阶段 SUS stage III		
1↑	脱氧核糖核酸酶-1 样蛋白 deoxyribonuclease-1-like	9.3994	4.64E-13
2↑	神经源性轨迹切口蛋白同源物 neurogenic locus notch protein homolog	9.1236	1.69E-04
3↑	成对类同源框转录因子 HpPitx paired superclass homeobox transcription factor HpPitx	8.9911	5.95E-04
4↑	IgG-Fc 片段结合蛋白 IgGFc-binding protein	4.017	0
5↑	C 型凝集素域家族 19 成员 A C-type lectin domain family 19 member A	3.9615	9.21E-16
6↑	黏蛋白 5AC mucin-5AC	3.8813	7.13E-33
7↑	WAS/WASL 相互作用蛋白家族成员 1 样蛋白 WAS/WASL- interacting protein family member 1-like	3.7744	7.66E-08
8↑	纤连蛋白 III 型域蛋白 7 样蛋白 fibronectin type III domain-containing protein 7-like	3.5849	4.09E-11
9↑	磷脂-2-酰基水解酶-2 phosphatidylcholine 2-acylhydrolase 2	3.3470	2.97E-05
10↑	α-N-乙酰半乳糖胺特异性凝集素 α-N-acetylgalactosamine-specific lectin	3.2153	3.45E-27
1↓	FER-1 相关蛋白样蛋白 FER-1-related-like	–8.7923	6.82E-04
2↓	自分泌增殖阻遏蛋白 A 样蛋白 autocrine proliferation repressor protein A-like	–7.3433	1.61E-06
3↓	Sushi, von Willebrand 因子, EGF 和五聚域包含蛋白 1 Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1(SVEP1)	–4.6809	1.04E-05
4↓	前蛋白转化酶枯草溶菌素 9 proprotein convertase subtilisin/kexin type 9	–4.3985	6.34E-24
5↓	酪氨酸蛋白激酶 Src42A tyrosine-protein kinase Src42A	–3.4285	7.27E-06
6↓	溶质载体有机阴离子转运蛋白家族成员 4A1 样蛋白 solute carrier organic anion transporter family member 4A1-like	–3.3183	4.47E-07
7↓	唾液腺分泌蛋白 1 salivary gland secretion 1	–2.9162	1.12E-45
8↓	MIF4G 域 1 核仁蛋白 nucleolar protein with MIF4G domain 1	–2.4703	2.10E-03
9↓	过氧化物酶体的生物合成因子 10 样蛋白 peroxisome biogenesis factor 10-like	–2.4703	2.10E-03
10↓	不育 α 基域包含蛋白 3 sterile α motif domain-containing protein 3	–2.4041	9.45E-05

注: ↑代表上调表达; ↓代表下调表达; FDR: 错误发现率; “0”代表 *P* value < 1E-238.

Note: ↑ represents up-regulated; ↓ represents down-regulated; FDR: false discovery rate; “0”means *P* value < 1E-238.

表 3 仿刺参“化皮”不同阶段的病变和正常体壁组织间差异表达基因 KEGG 富集分析
Tab. 3 Significantly enriched pathway in DEGs between SUS and normal body walls during skin ulceration syndrome (SUS) progression

通路 ID pathway ID	通路条目 pathway term	上调/下调 up/ down	Q 值 Q value
	“化皮” I 阶段 SUS stage I		
ko04080	神经活性配体受体相互作用 neuroactive ligand-receptor interaction	Up	1.08 E-04
ko00061	脂肪酸生物合成 fatty acid biosynthesis	Up	5.64 E-04
ko04610	补体与凝血级联 complement and coagulation cascades	Down	8.09E-08
ko04512	细胞外基质受体相互作用 ECM-receptor interaction	Down	2.19E-03
ko04640	造血细胞谱系 hematopoietic cell lineage	Down	1.71E-02
	“化皮” II 阶段 SUS stage II		
ko04145	过氧化物酶体 phagosome	Up	3.95E-02
ko04610	补体与凝血级联 complement and coagulation cascades	Down	2.87E-11
ko04512	细胞外基质受体 ECM-receptor interaction	Down	5.95E-09
ko04510	细胞黏附 focal adhesion	Down	1.86E-05
ko04640	造血细胞谱系 hematopoietic cell lineage	Down	3.69E-03
	“化皮” III 阶段 SUS stage III		
ko04610	补体与凝血级联 complement and coagulation cascades	Up	8.66E-09
ko04512	细胞外基质受体 ECM-receptor interaction	Up	2.55E-08
ko04510	细胞黏附 focal adhesion	Up	2.43E-04
ko04330	Notch 信号通路 notch signaling pathway	Up	2.35E-03
ko04141	内质网蛋白加工 protein processing in endoplasmic reticulum	Up	6.41E-03
ko04320	背腹轴形成 dorso-ventral axis formation	Up	1.63E-02
ko04610	补体与凝血级联 complement and coagulation cascades	Down	1.23 E-03

与凝血级联通路在“化皮” III 期既有上调基因又有下调基因。

2.3 仿刺参“化皮”过程关键差异表达基因调控网络构建

利用 KEGG 富集分析筛选出仿刺参“化皮” 3 个阶段均发生显著变化的信号通路: 细胞外基质受体相互作用(ECM-receptor interaction)通路和细胞黏附(focal adhesion)通路, 将通路中的相关基因列表导入 Cytoscape3.2.1 软件, 对仿刺参“化皮”相关差异表达基因进行可视化构网(图 3)。结果显示 *FcGBP* 基因在“化皮” 3 个阶段均参与到发生显著变化的信号通路。此外, TN 蛋白家族基因也在“化皮”不同阶段参与到发生显著变化的信号通路。

2.4 差异表达基因的 qRT-PCR 验证

为了验证 RNA-Seq 分析结果的可靠性, 选取 *FIBCD1*、*FcGBP*(在“化皮” I 期下调表达, 而在“化皮” III 期上调表达)、*CFH*、*TIMP1* 和 *GalNAc* 基因进行 qRT-PCR 验证。结果表明, 5 个基因在仿刺参“化皮”不同阶段表达趋势与 RNA-Seq 结果一致(图 4); 皮尔逊相关系数 r 值为 0.7714, 证明 RNA-Seq 分析结果准确可靠。

3 讨论

仿刺参“化皮病”的发生发展是一个多基因调控、多因素参与、动态的复杂过程, 其发病机制尚未阐明。在病原菌感染后, 有些个体体壁开始出现溃疡, 从一个或几个白色斑点开始, 并逐渐扩大, 直至大面积溃烂, 最后死亡, 整个过程持续 1~11 d[2]。对于“化皮病”个体, “化皮”只是外观看到的一种现象, 从病原菌感染开始产生溃疡至大面积溃疡, 仿刺参“化皮”过程中病变体壁组织的基因表达存在多样的动态表达模式[23]。为了研究仿刺参“化皮”过程中的分子调控机制, 除了要了解“化皮”不同阶段的病变组织与健康个体健康组织之间基因表达的差异, 还要了解“化皮”不同阶段同一个体中病变与正常组织之间基因表达的差异, 通过对这两种差异的比较分析, 从而更加

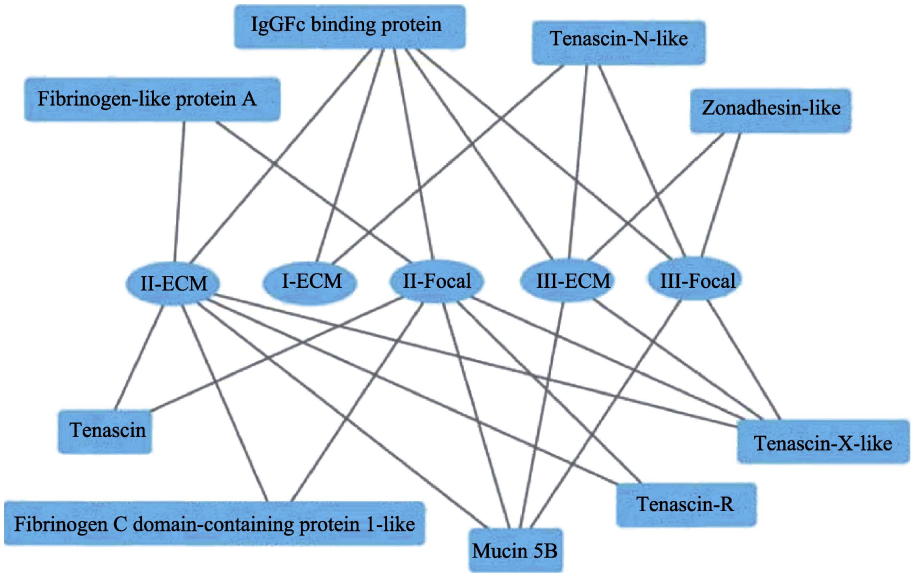

图 3　仿刺参“化皮”不同阶段差异表达基因调控网络构建

椭圆形框代表发生显著变化的信号通路; 长方形框代表差异表达基因; 椭圆形框中 I、II、III 代表“化皮” 3 个阶段.

Fig. 3　Construction of regulatory network for skin ulceration syndrome (SUS) progression in *Apostichopus japonicus*

Oval frame represents significantly enriched pathway; Rectangular frame represents DEGs; I, II, III in the oval frame represent three SUS stages.

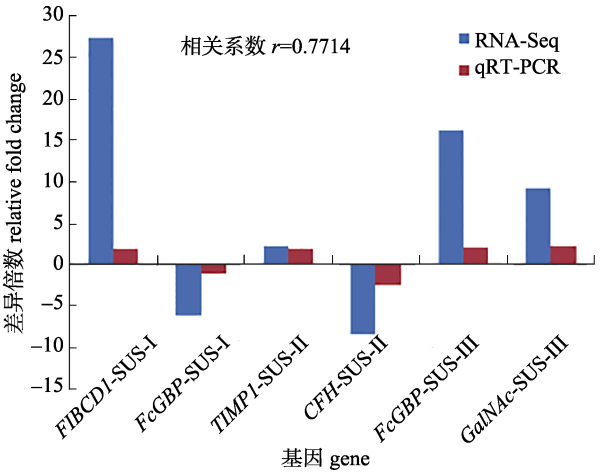

图 4　差异表达基因的 qRT-PCR 验证

Fig. 4　Validation of differentially expressed genes using qRT-PCR

深入地阐明不同阶段关键基因在仿刺参“化皮”过程中的作用、基因交互作用的分子机制与信号传导通路的调控机理。

仿刺参“化皮”不同阶段病变与正常体壁组织之间差异表达基因的 KEGG 富集分析结果显示, 补体与凝血级联通路(complement and coagulation cascades)在“化皮” 3 个阶段都显著改变, 尤其值得关注。补体系统参与机体的特异性和非特异性免疫机制, 通过经典途径、旁路途径和凝集素途径被激活, 从而发挥调理吞噬、介导炎症、免疫调节及清除免疫复合物等多种生物学效应。补体 C3 是补体系统中的重要分子, 是补体经典途径和旁路途径的中心环节。B 因子(Bf)是启动补体系统旁路途径的重要因子, 研究表明补体 C3、Bf 在仿刺参免疫系统中起着重要作用[8-10, 32]。与健康个体体壁组织相比, 补体成分 C3、Bf 基因在仿刺参“化皮”不同阶段病变体壁组织中高水平表达[23], 本研究中“化皮”不同阶段病变与正常组织之间差异表达基因分析结果表明, 补体 C3 基因在“化皮” II 期低水平表达, 补体 Bf 基因在“化皮”3 个阶段均高水平表达(结果未显示)。此外, 该信号通路中的调控因子 α2-巨球蛋白(alpha-2-macroglobulin, A2m)在“化皮病”仿刺参体腔细胞中低水平表达[19]。A2m 蛋白可作一种补体样调理素(complement-like opsonin)促进疟蚊(*Anopheles gambiae*)血细胞对革兰氏阴性细菌的吞噬作用[33]。最近, Zhong 等[34]和翟钰等[35]采用高通量测序筛选出与刺参补体 C3 相关的 miRNAs。补体信号通路在仿刺参“化皮”过程中的作用及其转录后的调控机制值得进一步深入研究。

细胞黏附(focal adhesion)和细胞外基质受体(ECM-receptor interaction)这两条通路在仿刺参“化皮”不同阶段的病变体壁组织与健康个体体壁组织之间也发生了显著的改变[23]。参与到这两条通路中的*FcGBP*基因在“化皮”不同阶段病变与正常组织之间, 以及“化皮”不同阶段的病变体壁组织与健康个体体壁组织之间[23]的表达都发生显著性的改变。FcGBP 是免疫球蛋白 IgG 的 Fc 段结合蛋白, 在胃、肠道黏膜层等富含黏液分泌细胞的组织器官中起到细胞保护和抗炎作用[36]。在人类早期溃疡性结肠炎隐窝组织中 *FcGBP* 上调表达[37]。此外, Tenascin 蛋白家族基因也在“化皮”不同阶段参与到发生显著变化的信号通路。Tenascin 是存在于细胞外基质中的一个蛋白家族。在仿刺参再生细胞外基质重塑的过程中, Aj-tenascin 基因表达水平发生显著改变[38]。在人类疾病研究中, TN 与肿瘤的浸润转移[39-40]、皮肤创伤后的愈合[41]以及溃疡组织的愈合[42-43]相关。仿刺参“化皮病”发生的重要标志是体壁出现白色溃疡斑点, 在仿刺参体壁真皮结缔组织的细胞间充填着大量的细胞外基质, TN 蛋白家族基因在病变与正常组织之间的差异表达提示其在仿刺参“化皮病”的发生发展中起到重要的调控作用。

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Identification of genes associated with skin ulceration syndrome in *Apostichopus japonicus* based on RNA Sequencing

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Abstract: In recent years, diseases caused by bacteria, viruses, and protozoa have severely limited the development of the sea cucumber (*Apostichopus japonicus*) aquaculture industry. Among such diseases, skin ulceration syndrome (SUS) has become the most universal and serious, owing to its high mortality rates. Therefore, the identification and analysis of key genes associated with "skin ulceration" and corresponding signal pathways are important for establishing the molecular mechanism of SUS. We previously analyzed the gene expression and transcriptome of three-stage SUS progression (SUS-I, SUS-II, SUS-III) in *A. japonicus* that had been challenged by *Vibrio splendidus*. Here, we further investigated the occurrence of differentially expressed genes (DEGs) among ulcerative and normal body wall (BW) samples from the same individuals at three stages of SUS progression. The R-Bioconductor package (R-2.15.3) was used to perform principal component analysis and Venn diagrams of these DEGs. KEGG enrichment analysis was carried out based on an algorithm (refer to materials and methods 1.2), using the entire transcriptome set as the background and a cutoff value of $Q \leqslant 0.05$. The regulatory network for SUS progression in *A. japonicus* was constructed using Cytoscape 3.2.1.

PCA analysis indicated that the number of DEGs among the ulcerative and normal BW samples was smallest at SUS stage II and that the gene expression profiles at SUS stages I and III were similar. Venn diagram analysis indicated that the 497, 59, and 433 unique DEGs were expressed at stage I, II and III of SUS progression, respectively. Only 28 DEGs were co-expressed in all three stages. KEGG enrichment analysis indicated that the "Complement and coagulation cascades" and "ECM-receptor interaction" pathways were significantly enriched throughout all three stages of SUS progression. The important SUS-related DEGs, including the FcGBP and TN family genes, were identified by constructing a regulatory network. Using qRT-PCR, five representative DEGs were selected to validate the sequencing results. The Pearson's correlation coefficient (r) was 0.7714, which confirmed the consistency and accuracy of the two approaches.

In sea cucumbers, SUS is characterized by apparent white skin ulcers, as well as by complicated molecular regulation. The significantly affected signal pathways detected among the ulcerative and normal BW in the same individuals at three stages of SUS progression, such as the "Focal adhesion" and "ECM-receptor interaction" pathways, have also been observed in the ulcerative BW of SUS-affected individuals (including SUS stages I, II, and III), when compared to healthy individuals in our previous studies. The FcGBP gene involved in these two pathways was worthy of further exploration. FcGBP is an Fc fragment of the IgG binding protein in fluids secreted by cells of the stomach and intestinal mucosa layer and might play a role in cell protection and anti-inflammation. FcGBP was up-regulated in crypts of early stage ulcerative colitis in human. In addition, Tenascins are extracellular matrix glycoproteins which can regulate cell adhesion, migration, proliferation and differentiation. The expression of the Aj-TN gene changed significantly during the process of extracellular matrix remodeling during sea cucumber regeneration. In human disease studies, TN proteins are associated with tumor metastasis, skin wound healing, and ulcer healing. The expression of TN-family genes in ulcerative and normal BW was significantly different during SUS progression, which suggests that these genes play important roles in the onset and development of SUS in sea cucumber. These results will be useful in developing strategies for preventing bacterial SUS in sea cucumber.

Key words: *Apostichopus japonicus*; skin ulceration; differentially expressed gene; regulatory network

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