内关穴位埋针心肌缺血损伤小型猪血管生长功能性基因的表达谱**☆

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文章亮点：①选取中国小型猪之一贵州小型猪作为实验动物，在针灸领域应用较少。②从血管新生角度研究针刺内关防治心肌缺血损伤机制。③实验发现心肌缺血以及穴位埋针后的干预均与血管生长因子基因差异表达有关。

关键词：内关；膈俞；穴位埋针；针灸；小型猪；基因芯片；心肌缺血模型；血管生成；组织构建

摘要

背景：内关针刺可上调相关血管生长因子的表达，但血管新生相关生长因子较多，针对部分因子研究有局限性。

目的：观察心肌缺血损伤小型猪血管生长功能性基因表达谱特征及内关穴位埋针的干预效应。

方法：将 32 只小型猪随机分为 4 组，模型组采用左冠状动脉前降支结扎法建立小型猪心肌缺血模型，内关组和膈俞组分别在造模基础上进行内关、膈俞穴埋针治疗，假手术组穿线不结扎。埋针 7 d 后取各组小型猪的缺血心肌组织进行实验。

结果与结论：应用 Q Series 血管生长功能基因组表达谱芯片共检测到 96 个与血管生成功能密切相关的基因。与模型组比较，内关组显著上调基因 17 个，下调基因 3 个，膈俞组显著上调基因 14 个，下调基因 2 个。就其基因功能分类而言，内关组、膈俞组与模型组比较差异表达基因各有其自身特点，但特异的促进因子和抑制因子、血小板衍生生长因子及受体、成纤维细胞生长因子及受体、生长因子及受体以及其他细胞生长因子，细胞因子和化学增活素、基质蛋白酶及抑制剂以及其他相关基因是各组共有的，其中以血管生长因子及受体最多。说明心肌缺血以及穴位埋针后的干预均与血管生长因子差异表达有关，但内关埋针与膈俞埋针作用不完全相同。

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Gene-expression profiles about myocardial ischemia injury miniature pig's growth function and intervention effects of needle-embedding therapy at *Neiguan*

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Abstract

BACKGROUND: Acupuncture at *Neiguan* (PC6) can up-regulate relative expressions of vascular growth factors. Nonetheless, there are plenty of growth factors related to angiogenesis, and researches have limitations if just focusing on a part of these factors.

OBJECTIVE: To observe characteristics on gene-expression profiles about myocardial ischemia injury miniature pig's growth function and intervention effects of needle-embedding therapy at *Neiguan*.

METHODS: Thirty-two miniature pigs were randomized into four groups. Afterwards, ligation of the left anterior descending coronary artery was performed to establish myocardial ischemia models. Meanwhile, pigs in sham operation group only underwent threading without ligation, while needle-embedding therapy at *Neiguan* and *Geshu* (BL17) was conducted in *Neiguan* group and *Geshu* group, respectively. Then, ischemic myocardial tissue was extracted for experiments after 7 days needle-embedding therapy.

RESULTS AND CONCLUSION: A total of 96 genes were detected that were closely related to the function of angiogenesis by using Q Series Angiogenesis' functional genomics expression profiles. Compared with the model group, there were 17 significantly up-regulated genes and 3 down-regulated genes in the *Neiguan* group; and 14 significantly up-regulated genes and 2 down-regulated genes in the *Geshu* group. As to classifications of gene function, differentially expressed genes had their own features when comparing differences expressions among *Neiguan* group, *Geshu* group and model group. Nevertheless, distinctive promoting factor and inhibitor, platelet derived growth factor and receptor, fibroblast growth factor and receptor, growth factor and receptor, other cell growth factors, cytokine and chemokines active element, matrix metalloproteinase and inhibitor, as well as other relative genes were shared between groups; among them, angiogenesis factors and receptors were the most. It indicates that intervention for myocardial ischemia and needle-embedding therapy are related to angiogenesis factors, but the action mechanism of needle-embedding therapy at *Neiguan* is different from that at *Geshu*.

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0 引言

大量临床和实验研究表明内关是治疗心肌缺血、改善心肌功能的重要穴位[1]。前期研究表明内关埋针可以显著上调与血管新生相关的多种生长因子，如血管内皮生长因子、转化生长因子β3及成纤维细胞生长因子基因和蛋白的表达[2-3]。实验继续用内关穴位埋针为干预手段，以缺血心肌为研究对象，选用Q Series血管生长功能基因组基因芯片为技术手段，通过现代生物信息学分析，研究缺血性损伤心肌血管生长因子相关分子功能类别基因表达整体特征，探讨内关埋针促进血管生成的可能机制。

1 材料和方法

设计：随机对照动物实验。

时间和地点：于2006-08在贵阳中医学院完成。

材料：

主要仪器及试剂：

试剂及仪器	来源
MHJ-ⅢB2 呼吸麻醉机	上海泰益医疗仪器设备有限公司
XDH-3B 型心电图机	上海医用电子仪器有限公司
胸腔引流管	长沙回春医疗器械公司
DYY-8 型稳压稳流电泳仪	上海琪特分析仪器有限公司
FeroTec Gradien PCR 扩增仪	杭州大和热磁电子有限公司
DK-8D 型电热恒温水槽	上海森信实验仪器有限公司
图像分析软件	Imagene 公司
Q Series 血管生长基因芯片 (HS009)、GEAhyb 杂交液，10×RT 缓冲液，缓冲液 A	SuperArray Bioscience
Trizol 试剂盒，无 RNA 酶的糖原	Invitrogen life technologies
RNeasy Mini Kit	Qiagen
RNA 酶抑制剂，M-MLV 反转录酶	Promega
生物素标记 dUTP	Roche

实验动物：贵州小型猪32只，雌雄不限，7-10月龄，体质量25-35 kg，由贵阳中医学院动物研究所提供。

方法：

实验动物分组与心肌缺血模型的制备：将32只小型猪随机分为4组：假手术组、模型组、内关组和膈俞组，每组8只。除假手术组外均参照文献[1]进行造模，于左冠状动脉前降支下1/3交界稍偏上处穿线，预结扎15-20 min后，正式结扎，立即心电图监测，发现Ⅱ导联ST段上移＞0.05 mV，心尖区颜色变暗，说明造模成功。假手术组仅开胸、穿线但不结扎。

埋针方法及干预：取穴采用模拟人体经穴法结合比较解剖学的方法，对贵州小型猪进行穴位定位，由贵阳中医学院解剖教研室协助完成。内关：尺骨鹰嘴结节水平线至桡骨远端背侧关节面凹陷之水平线，桡骨远侧端上1/6处，前肢掌侧面尺、桡骨之间。膈俞：第7肋间隙背最长肌外侧沟中。预先将8 mm×40 mm的圆利针(兽医专用)针身剪断为1.5 cm，仿皮内针弯成"7"字型，将小型猪穴区鬃毛剪去，消毒，用持针器将圆利针刺入穴位1 cm深后，用持针器头向下按压圆利针，检查并确定圆利针全部进入小型猪体内，留置时间为7 d。

造模后，内关组和膈俞组分别行双侧内关穴、膈俞穴穴位埋针。模型组和假手术组不进行干预。

缺血心肌组织取材：埋针治疗7 d后，取各组小型猪，麻醉处死后取出心脏，切掉左右心房、右心室和左心室结扎部位以上部分，与房室环平行横切心肌(约5 mm×1 mm×1 mm)，一部分立即置于液氮中，并转存于-80 ℃冰箱。

Gene Array基因芯片检测：取各组心肌组织100 mg，Trizol法提取总RNA，紫外分光光度计测A_{260}/A_{280}比值介于1.8-2.0之间，根据A_{260}计算总RNA含量，1%琼脂糖电泳观察总RNA的质量。Rneasy Kit纯化RNA，M-MLV反转录酶合成cDNA，生物素标记dUTP合成探针，预杂交后，将变性的cDNA探针加入0.75 mL预热的GEAprehyb中混匀，弃杂交管中GEAprehyb

溶液，加入含探针的GEAhyb混合液，6 r/min 60 ℃杂交过夜。充分洗膜，封闭，弃封闭液，加入2 mL结合缓冲液链亲和素偶联的碱性磷酸酶，在杂交仪中轻轻摇动孵育10 min，洗膜，化学发光法检测信号。X射线胶片曝光后，将胶片上的图像用扫描仪扫描并转换为灰度TIFF格式的图片文件保存。运行ScanAlyze软件，将灰度TIFF格式图片的点阵转化为数字型数据，将此原始数据储存为Microsoft Excel文件。每张芯片都点有负对照(PUC18DNA 和空白)，包括β-actin，GAPDH，Cyclophilin A和核糖体蛋白L13a，管家基因为GAPDH。

芯片数据分析：使用芯片配套软件GEArray Analyzer，原始数据将首先被减掉背景最小值，继而用管家基因来进行校正，校正后的数据用来进行样品间基因转录的相对丰度分析，以比较不同标本特定基因表达水平的差异。当两组基因表达水平比值≥2时，表示该基因表达显著上调，当两组基因表达水平比值≤0.5时，表示该基因表达显著下调。

主要观察指标：各组缺血心肌组织血管生长功能基因表达情况。

2 结果

2.1 实验动物数量分析
实验共纳入32只小型猪，均进入结果分析。

2.2 各组小型猪血管新生相关生长因子基因表达丰度
读图顺序为，自左到右，自上到下。每张图的芯片杂交斑点序号均为1，2，3……112，前96是血管新生相关生长因子基因，而从97到112为负对照和管家基因，包括β-actin，GAPDH，Cyclophilin A，核糖体蛋白L13a。不同芯片同一位置为同一基因，见图1。4张芯片分别代表假手术组、模型组、内关组和膈俞组血管生长因子各分子特异信号强度。
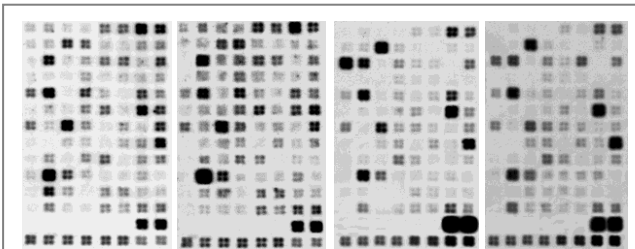
a: Sham operation group　b: Model group　c: *Neiguan* group　d: *Geshu* group

Figure 1　General view of abundance about mRNA on relative factors of angiogenesis in ischemic myocardial tissue of miniature pigs in each group
图 1　各组小型猪缺血心肌组织血管生长相关因子 mRNA 表达丰度图

2.3 各组小型猪血管新生相关生长因子基因差异表达情况

模型组与假手术组血管新生相关生长因子mRNA表达水平的比较：见表1，2，图2。

表 1　模型组和假手术组比较上调 2 倍以上的基因及其功能分类
Table 1　The genes and their function classification were up-regulated more than two folds in the model group compared with sham operation group

Function classification		GenBank	Name
Specific promoting factor and inhibitor		NM_001145	Angiogenic factors (ANG)
		NM_001146	Angiogenesis factors (ANGPT1)
Growth factor and receptor	Protein tyrosine kinase family	NM_004093	Protein tyrosine kinase B2 (EFNB2)
		NM_004444	Protein tyrosine kinase B4 (EPHB4)
	Fibroblast growth factor and its receptor	NM_002007	Fibroblast growth factor 4 (FGF4)
		NM_002009	Fibroblast growth factor 7 (FGF7)
		NM_000604	Fibroblast growth factor receptor 1 (FGFR1)
		NM_000141	Fibroblast growth factor receptor 2 (FGFR2)
	Vascular endothelial growth factor and its receptor	NM_002253	Tyrosine kinase 3 receptor (KDR)
		NM_002632	Placental growth factor (PGF)
		NM_003376	Vascular endothelial growth factor (VEGF)
	Transforming growth factor and its receptor	NM_003239	Transforming growth factor β3 (TGFβ3)
	Other growth factors	NM_000072	CD36 antigen, platelet thrombin-sensitive protein (CD36)
		NM_001400	G protein coupled receptor 1 (EDG1)
		NM_001963	Epidermal growth factor (EGF)
Cytokines and chemokines active element		NM_002176	Interferon β1 (IFNB1)
		NM_000619	Interferon γ(IFNG)
		NM_003873	Neuropilin 1(NRP1)
Matrix proteins, proteases and their receptors		NM_006988	Disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1)
		NM_007037	Disintegrin and metalloproteinase with thrombospondin motifs 8 (ADAMTS8)
		NM_007112	Platelet thrombin-sensitive protein 3 (THBS3)
		NM_003248	Platelet thrombin-sensitive protein 4 (THBS4)
Other related genes		NM_000603	Nitric oxide synthase 3 (NOS3)

从基因芯片探针表达数量结果看，模型组与假手术组比，差异表达上调2倍及2倍以上的基因23个，归纳起来主要为特异性的促进因子和抑制因子、生长因子及受体、细胞因子和化学增活素、基质蛋白酶及其受体、及其他相关基因；差异表达下调0.5倍及0.5以上的基因12个，主要为生长因子及受体、转录因子、细胞因子和化学增活素、基质蛋白酶及其受体及黏附分子。

表2 模型组和假手术组比较下调 0.5 倍以上的基因及其功能分类

Table 2 The genes and their function classification were down-regulated more than 0.5 fold in the model group compared with sham operation group

Function classification		GenBank	Name
Growth factor and receptor	Fibroblast growth factor and its receptor	NM_002006	Fibroblast growth factor 2 (FGF2)
	Platelet-derived growth factor and its receptor	NM_002607	Platelet-derived growth factor alpha polypeptide (PDGFA)
		NM_003236	Transforming growth factor alpha (TGFα)
	Transforming growth factor and its receptor	NM_003242	Transforming growth factor β receptor 2 (TGFβR2)
		NM_003243	Transforming growth factor β receptor 3 (TGFβR3)
	Other growth factors	NM_000601	Hepatocyte growth factor (HGF)
Transcription factor		NM_005900	Mothers against decapentaplegic homolog 1 (MADH1)
Matrix proteins, proteases and their receptors		NM_004530	Matrix metalloproteinase 2 (MMP2)
		NM_003255	Tissue inhibitor of metalloproteinase 2 (TIMP2)
Adhesion molecule		NM_000442	Platelet/epithelial cell adhesion molecule (PECAM1)
Cytokines and chemokines active element		NM_002825	Heparin-binding growth factor (PTN)
		NM_000594	Tumor necrosis factor (TNF)
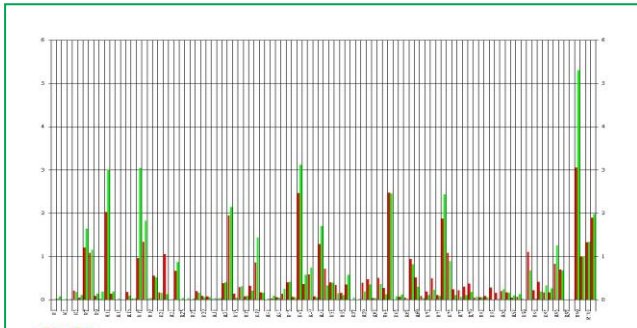
Exp.1: Sham operation group; Exp.2: Model group

Figure 2 mRNA expression of growth factors related to angiogenesis in the model and sham operation groups

图2 模型组与假手术组血管新生相关生长因子 mRNA 的表达水平

表3 内关组和模型组比较上调 2 倍以上的基因及其功能分类

Table 3 The genes and their function classification were up-regulated more than two folds in the Neiguan group compared with model group

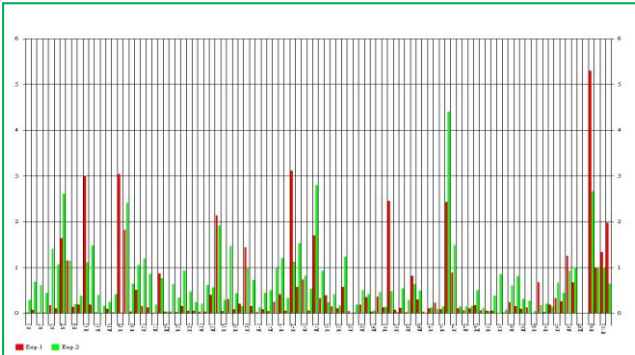| Function classification		GenBank	Name
Specific promoting factor and inhibitor		NM_001145	Angiogenic factors (ANG)
		NM_001146	Angiogenesis factors (ANGPT1)
Growth factor and receptor	Platelet-derived growth factor and its receptor	NM_002607	Platelet-derived growth factor alpha polypeptide (PDGFA)
	Fibroblast growth factor and its receptor	NM_002006	Fibroblast growth factor 2 (FGF2)
		NM_004612	Transforming growth factor β receptor 1 (TGFβR1)
		NM_003242	Transforming growth factor β receptor 2 (TGFβR2)
	Vascular endothelial growth factor and its receptor	NM_002253	Tyrosine kinase 3 receptor (KDR)
		NM_003376	Vascular endothelial growth factor (VEGF)
	Transforming growth factor and its receptor	NM_003239	Transforming growth factor β3 (TGFβ3)
	Other growth factors	NM_000601	Hepatocyte growth factor (HGF)
		NM_001400	G protein coupled receptor 1 (EDG1)
		NM_001963	Epidermal growth factor (EGF)
Cytokines and chemokines active element		NM_002176	Interferon β1 (IFNB1)
		NM_000619	Interferon γ(IFNG)
		NM_003873	Neuropilin 1 (NRP1)
Matrix proteins, proteases and their receptors		NM_006988	Disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1)
		NM_007037	Disintegrin and metalloproteinase with thrombospondin motifs 8 (ADAMTS8)

表4 内关组和模型组比较下调 0.5 倍以上的基因及其功能分类

Table 4 The genes and their function classification were down-regulated more than 0.5 fold in the Neiguan group compared with model group

Function classification		GenBank	Name
Growth factor and receptor	Protein tyrosine kinase family	NM_004444	Protein tyrosine kinase B4 (EPHB4)
	Vascular endothelial growth factor and its receptor	NM_002632	Placental growth factor (PGF)
Other related genes		NM_000603	Nitric oxide synthase 3 (NOS3)

内关组与模型组血管新生相关生长因子mRNA表达水平的比较：见表3，4，图3。

从基因芯片探针表达数量结果看，内关组与模型组比，差异表达上调2倍及2倍以上的基因17个，归纳起来主要为特异性的促进因子和抑制因子、生长因子及受体、细胞因子和化学增活素、基质蛋白，蛋白酶及其受体；差异表达下调0.5倍及0.5以上的基因3个，主要为生长因子及受体和其他相关基因。

膈俞组与模型组血管新生相关生长因子mRNA表达水平的比较：见表5，6，图4。

从基因芯片探针表达数量结果看，膈俞组与模型组比较，差异表达上调2倍及2倍以上的基因14个，归纳起

来主要为特异性的促进因子和抑制因子、生长因子及受体、细胞因子和化学增活素、基质蛋白，蛋白酶及其受体；差异表达下调0.5倍及0.5以上的基因2个，为生长因子及其受体基因。

Exp.1: Model group; Exp.2: *Neiguan* group

Figure 3　mRNA expression of growth factors related to angiogenesis in the model and *Neiguan* groups
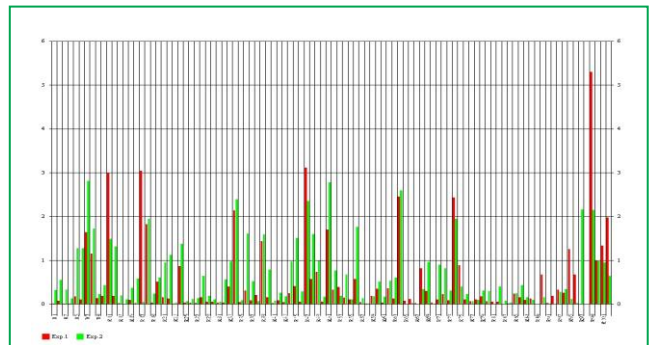图 3　模型组与内关组血管新生相关生长因子 mRNA 的表达水平

表 5　膈俞组和模型组比较上调 2 倍以上的基因及其功能分类
Table 5　The genes and their function classification were up-regulated more than two folds in the *Geshu* group compared with model group

Function classification		GenBank	Name
Specific promoting factor and inhibitor		NM_001145	Angiogenic factors (ANG)
		NM_001146	Angiogenesis factors (ANGPT1)
Growth factor and receptor	Platelet-derived growth factor and its receptor	NM_002607	Platelet-derived growth factor alpha polypeptide (PDGFA)
	Fibroblast growth factor and its receptor	NM_002006	Fibroblast growth factor 2 (FGF2)
		NM_000604	Fibroblast growth factor receptor 1 (FGFR1)
		NM_000141	Fibroblast growth factor receptor 2 (FGFR2)
	Transforming growth factor and its receptor	NM_003239	Transforming growth factor β3 (TGFβ3)
	Vascular endothelial growth factor and its receptor	NM_003376	Vascular endothelial growth factor (VEGF)
	Other growth factors	NM_00060	Hepatocyte growth factor (HGF)
		NM_001963	Epidermal growth factor (EGF)
Cytokines and chemokines active element		NM_002176	Interferon β1 (IFNB1)
		NM_000619	Interferon γ (IFNG)
Matrix proteins, proteases and their receptors		NM_006988	Disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1)
		NM_007037	Disintegrin and metalloproteinase with thrombospondin motifs 8 (ADAMTS8)

表 6　膈俞组和模型组比较下调 0.5 倍以上的基因及其功能分类
Table 6　The genes and their function classification were down-regulated more than 0.5 fold in the *Geshu* group compared with model group

Function classification		GenBank	Name
Growth factor and receptor	Protein tyrosine kinase family	NM_004444	Protein tyrosine kinase B4 (EPHB4)
	Vascular endothelial growth factor and its receptor	NM_002632	Placental growth factor (PGF)

Exp.1: Model group; Exp.2: *Geshu* group

Figure 4　mRNA expression of growth factors related to angiogenesis in the model and *Geshu* groups
图 4　模型组与膈俞组血管新生相关生长因子 mRNA 的表达水平

3　讨论

自1999年基因芯片技术应用于心血管系统的研究以来[4-5]，已经在该领域进行了多组织、多个层次的研究，并取得进展，如用AffymetrixRAT 230A基因表达谱芯片、cDNA芯片基因芯片技术研究心肌缺血或缺血/再灌注后心肌组织细胞基因表达谱[6-7]。另外有研究利用基因芯片技术分析caspase基因及其相关序列在缺血心肌细胞中的表达情况，筛选得到了3条大鼠心肌细胞差异表达基因，初步证实了电针可以调节细胞内凋亡调控因子caspase的表达[8]，同时该研究组观察电针神门穴后心肌组织G蛋白基因表达，分析电针对心肌缺血保护的分子作用机制[9]。但以上研究中未从血管新生相关基因表达谱角度探讨针刺对缺血心肌保护的分子机制。由此实验在前期工作的基础上，重复验证内关埋针具有改善缺血心肌功能的同时，进一步研究内关埋针促进血管生成的相关基因表达谱特征,通过生物学功能性芯片共检测到96个与血管生成功能密切相关的基因，具有高针对性，避免了高通量基因芯片数据过多，难以分析的缺点,因而增强了结果的可信度。
血管新生是指当血管受到局部缺氧、缺血、机械牵拉

及炎症等刺激后，已分化成熟的内皮细胞就被激活，局部血管舒张，血管通透性增加，血管外纤维蛋白聚集，基底膜分裂，内皮细胞朝着刺激来源的方向迁移入组织间隙内，既而增生，细胞内空泡管腔形成，细胞周围基底膜生成，最终在原有血管的基础上生出新的血管。包含有3种形式，其中血管新生诱导因子作用下毛细血管芽生式新生最为常见，研究发现此类因子有多种，包括血管内皮生长因子、成纤维细胞生长因子、干细胞生长因子、血管形成素1等[10]，但除上述因子以外，还有哪些与血管生成功能有关的因子参与到缺血心肌后的血管生成？

　　实验通过生物学功能性芯片共检测到96个与血管生成功能密切相关的基因，根据基因芯片的荧光强度获得差异表达基因，分别将模型组与假手术组比较、内关组与模型组比较、膈俞组与模型组比较。其中模型组与假手术组比较的差异表达基因35条，上调23条，下调12条；内关组与模型组比较的差异表达基因20条，上调17条，下调3条；膈俞与模型组比较上调14条，下调2条，内关组的差异表达基因显著多于膈俞组，但有相同表达基因，只是其表达信号绝对值不相同，即表达量不同，提示内关埋针与膈俞埋针作用机制和途径可能不同。就其基因功能分类而言，内关组、膈俞组与模型组比较差异表达上调基因各有其自身特点，在3个组比较中共有的差异表达基因(包括上调和下调)为ANG、ANGPT1、PDGFA、FGF2、FGFR1、FGFR2、TGFβ3、VEGF、HGF、EGF、IFNB1、IFNG、ADAMTS1、ADAMTS8，有其自身特点，但特异的促进因子和抑制因子、血小板衍生生长因子及受体、成纤维细胞生长因子及受体、血管生长因子及受体以及其他生长因子，细胞因子和化学增活素、基质蛋白酶及抑制剂是组间共有的，其中以血管生长因子及受体所占比例最大，由此可看出心肌缺血以及内关穴位埋针后的干预均主要与血管生长因子及受体的表达差异有关，但为了进一步明确上述血管新生相关生长因子的基因表达及其蛋白表达情况，以及在各组间的差异如何，需要进一步用基因和蛋白检测手段验证，以揭示心肌缺血及内关埋针治疗的机制。

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