论　著　　·临床研究·

Toll样受体基因组特征与直肠癌临床病理及免疫参数的相关性分析

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[摘要]　**目的**　探讨Toll样受体家族成员对直肠癌发生、预后和免疫特征的影响，以及其基因调节区遗传变异与直肠癌发病风险的关系。**方法**　使用GEPIA平台分析Toll样受体3(TLR3)、TLR4、TLR5和TLR7在直肠癌中的表达及其与预后的关系。从TCGA数据库获取直肠癌mRNA转录组数据和临床资料，分析TLR3表达与病理分期的关系。使用PCR-RFLP技术对TLR4 rs1927914、rs7869402多态进行基因分型，使用TaqMan探针法对TLR3 rs5743303、TLR5 rs1640816、TLR7 rs7869402多态进行基因分型。应用非条件logistic回归分析TLRs遗传变异与直肠癌发病风险的关系。使用ESTIMATE对直肠癌样本的免疫细胞进行评分，通过TIMER在线数据平台分析TLR3表达及拷贝数变异(CNV)与免疫细胞浸润的关系。GO功能富集分析TLR3相关差异基因的表达，基因集变异分析(GSVA)预测直肠癌中TLR3的调控网络。**结果**　TLR3在直肠癌组织中呈低表达，且其低表达与预后不良有关。与TLR3 rs5743303 AA基因型相比，AT或TT基因型携带者的直肠癌发病风险明显升高(*OR*=1.43，95%CI 1.07~1.91)。TLR4 rs1927914、TLR4 rs7869402、TLR5 rs1640816及TLR7 rs3853839多态不影响直肠癌的发病风险。分层分析结果显示，TLR3 rs5743303 AT或TT基因型可增加男性(*OR*=1.47，95%CI 1.01~2.11)、低年龄者(*OR*=1.64，95%CI 1.08~2.47)、吸烟者(*OR*=2.42，95%CI 1.37~4.38)和饮酒者(*OR*=2.70，95%CI 1.52~4.80)直肠癌的发病风险。ESTIMATE和TIMER分析结果显示，TLR3与免疫细胞浸润具有一定相关性。GO功能富集分析结果显示，TLR3主要与受体配体活性、抗原结合、免疫球蛋白受体结合、生长因子和细胞因子等相关功能的改变有关。GSVA分析结果显示，TLR3可通过调节细胞凋亡、自然杀伤细胞介导的细胞毒性、磷脂代谢及自噬调节等与代谢或免疫相关的途径而抑制直肠癌的发生发展。**结论**　TLR3与直肠癌的发生及预后相关，TLR3 rs5743303遗传变异可影响直肠癌的遗传易感性。

[关键词]　Toll样受体；单核苷酸多态性；免疫；直肠癌

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Correlation analysis between Toll-like receptor genomic characteristics and clinicopathological and immune parameters in rectal cancer

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[**Abstract**]　**Objective**　To investigate the effects of Toll-like receptors (TLR3/TLR4/TLR5/TLR7) on the development, prognosis and immune characteristics of rectal cancer and to explore the association of genetic variation in the regulation region of TLRs with the risk for rectal cancer. **Methods**　Gene expression profiling interactive analysis (GEPIA) platform was used to analyze the expression of TLRs in rectal cancer and its relationship with prognosis. Transcriptome data and clinical data of rectal cancer were downloaded from TCGA database and the correlation between TLRs expression and pathological stage was analyzed. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the genotype of TLR4 rs1927914 and rs7869402 polymorphism. TaqMan probe method was used to determine the genotypes of TLR3 rs5743303, TLR5 rs1640816 and TLR7 rs7869402 variants. The association of TLRs genetic variants with the rectal cancer risk was analyzed by unconditional logistic

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regression. The immune cells in rectal cancer samples were scored using ESTIMATE algorithm. Using TIMER online data platform, the relationship between both the expression of TLRs and copy number variation (CNV) of genes and immune infiltrated cells was evaluated. Gene function related to TLR3 expression was evaluated by GO function enrichment analysis, gene set variation analysis (GSVA) was used to predict the regulatory network of TLR3 in rectal cancer. **Results** TLR3 has a lower expression in rectal cancer tissues than in adjacent tissues, which was related to poor prognosis. Compared with the individuals carrying TLR3 AA genotype, the individuals with at least one TLR3 rs5743303 T allele had significantly higher risk of rectal cancer (*OR*=1.43, 95%CI 1.07-1.91). The study did not show that TLR4 rs1927914, TLR4 rs7869402, TLR5 rs1640816 and TLR7 rs3853839 polymorphism had effect on the risk for rectal cancer. After stratified analysis, the data showed that TLR3 rs5743303 AT or TT contributed the susceptibility to rectal cancer in males (*OR*=1.47, 95%CI 1.01-2.11), younger subjects (*OR*=1.64, 95%CI 1.08-2.47), smokers (*OR*=2.42, 95%CI 1.37-4.38) and drinkers (*OR*=2.70, 95%CI 1.52-4.80). ESTIMATE and TIMER analyses showed that TLR3 effected on the immune cell infiltration. The results of GO functional enrichment analysis showed that TLR3 was mainly related to changes in related functions such as receptor ligand activity, antigen binding, immunoglobulin receptor binding, growth factors and cytokines. GSVA results showed that TLR3 inhibited the development of rectal cancer by regulating metabolic or immune-related pathways, such as cell apoptosis, natural killer cell-mediated cytotoxicity, phospholipid metabolism and autophagy regulation. **Conclusion** TLR3 effects on the occurrence and prognosis of rectal cancer and rs5743303 polymorphism increase the susceptibility to rectal cancer.

[**Key words**] Toll-like receptor; single nucleotide polymorphism; immunity; rectal cancer

结直肠癌(colorectal cancer，CRC)是世界上第三大常见癌症，也是导致癌症患者死亡的第二大原因[1]。美国癌症协会的流行病学调查显示，直肠癌在CRC中占比达35%。作为先天免疫的重要组成部分，炎症是癌症发生和进展的标志性特征[2]。Toll样受体(Toll like receptors，TLRs)是特殊的模式识别受体(pattern recognition receptors，PRRs)，可诱导免疫细胞表面相关分子的表达而激活免疫应答途径，还可与相应配体结合激活炎症通路[3]。据统计，除环境危险因素(包括肥胖、缺乏运动、不良饮食、饮酒和吸烟)外，1/3的直肠癌是由遗传因素诱导发生的[4-5]，而单核苷酸多态性(single nucleotide polymorphism，SNP)在人类的可遗传变异中较为常见。调节区和3'非翻译区SNP可分别通过调节与转录因子或miRNA的结合，从而对靶基因调控产生影响[6]。因此，具有潜在功能的SNP可能会增加机体对癌症的易感性。有研究发现，TLR3调控区基因多态性与肠道病毒71型(EV71)重症感染的易感性相关[7]，而肠道感染也是CRC的诱因之一[8]。鉴于TLRs在肿瘤中的重要作用，本研究通过查阅文献并利用生物信息学方法筛选出5个潜在的功能SNP位点，探讨其对CRC发病风险的影响，以期为CRC的防治及早期诊断提供理论依据。

1 资料与方法

1.1 研究对象 本研究采用病例对照的研究策略。选取2008—2016年在华北理工大学附属唐山市工人医院和华北理工大学附属唐山市人民医院住院治疗的480例经组织病理学确诊且采血前未接受放化疗的原发性直肠癌患者设为病例组，另随机选取同期在唐山地区进行体检的480名健康者设为对照组，排除与病例组有血缘关系、有肿瘤家族史、接受放射治疗或者抗癌化学药物治疗的个体。本研究经华北理工大学伦理审查委员会批准(2019021)，所有参与者均签署知情同意书。

1.2 SNP筛选 从dbSNP及Ensembl数据库中提取TLR家族成员基因多态数据信息，筛选位于启动子区及3'非翻译区且在中国汉族人群中最小等位基因频率>0.05的遗传变异。使用TRANSFAC和SNPinfo Web Serve软件分别对筛选出的SNP进行转录因子结合和miRNA结合能力预测，最终确定研究位于启动子区的TLR3 rs5743303、TLR4 rs1927914、TLR5 rs1640816，以及3'非翻译区的TLR4 rs7869402、TLR7 rs3853839多态。

1.3 基因分型 使用DNA提取试剂盒(北京天根生化科技有限公司)提取外周血DNA。针对TLR3 rs5743303、TLR5 rs1640816和TLR7 rs3853839多态，使用TaqMan探针法进行基因分型。PCR反应体系包括1×Taq PCR MasterMix Ⅱ(美国ABI公司)2 μl、探针0.065 μl和基因组DNA 1 μl(>10 ng)，最后用双蒸水补至5 μl，使用ABI 7900HT Fast Real-Time PCR system进行检测。针对TLR4 rs1927914和rs7869402多态，使用聚合酶链反应限制性片段长度多态性(PCR-RFLP)技术进行基因分型。引物序列如下：TLR4 rs1927914上游5'-TAGCATGAGAAATGAGGAAGTAAGGG-3'，下游5'-GAGCTATGATGAGGATTGAAAATGTGG-3'；TLR4 rs7869402上游5'TGGGATCCCTCCCCTGTAGC-3'，下游5'-AGGAGCATTGCCCAACAGG-3'。PCR反应体系包括上下游引物各0.1 μl(10 mmol/L)，cDNA 1 μl(>10 ng)，2×Taq PCR Starmix 3 μl(北京康润诚业生物科技有限公司)，最后用双蒸水补至6 μl。

PCR扩增条件：94 ℃预变性3 min；94 ℃变性30 s，58 ℃退火30 s，72 ℃延伸30 s，进行30个循环；然后72 ℃延伸5 min。使用*Alu* Ⅰ和*Nsi* Ⅰ限制性核酸内切酶(NEB公司)对TLR4 rs1927914和rs7869402的PCR产物进行酶切。酶切产物经2.5%琼脂糖凝胶电泳进行基因型判断。为提高基因分型的准确度，随机选取10%的样本进行重复实验，结果一致。

1.4　统计学处理　利用GEPIA在线数据平台分析直肠癌中TLRs的表达及其与直肠癌预后的相关性，ESTIMATE算法分析TLR3表达与免疫细胞评分的相关性，TIMER数据库评估直肠癌TLR3表达及拷贝数变异与免疫细胞浸润的相关性。从TCGA数据库获取直肠癌患者的转录组数据和临床信息，利用WilcoxTest函数评估TLRs基因表达与病理分期的关系，KEGG通路和GO功能富集分析TLR3相关差异基因的表达[利用R语言中的基因集变异分析(GSVA)软件包完成]。采用SPSS 23.0软件进行统计分析。采用χ^2检验比较病例组与对照组研究对象的基本特征及基因型分布的差异，非条件logistic回归分析TLRs遗传变异与直肠癌发病风险的关系。所有统计检验均为双侧概率检验，P<0.05为差异有统计学意义。

2　结　　果

2.1　TLRs与直肠癌临床病理的关系　GEPIA在线数据平台分析结果显示，TLR3在直肠癌组织中的表达明显低于正常组织(P<0.05)，且其低表达与预后不良相关(HR=0.26，95%CI 0.094~0.87，P=0.021)。TLR4、TLR5和TLR7在直肠癌组织与正常组织中的表达差异无统计学意义，且与直肠癌预后无关(P>0.05)。TLRs的表达与直肠癌临床病理分期无相关性(P>0.05)(图1)。

2.2　TLRs遗传变异与直肠癌易感性的关系　两组年龄中位数均为60岁，年龄、性别以及吸烟和饮酒占比等基本资料比较，差异无统计学意义(P>0.05，表1)。

TLRs遗传变异各基因型分布与直肠癌易感性的关系如表2所示。病例组TLR3 rs5743303 AA、AT和TT基因型频率分别为69.8%(335/480)、26.7%(128/480)和3.5%(17/480)，对照组分别为76.7%(368/480)、20.8%(100/480)和2.5%(12/480)。非条件logistic回归分析结果显示，与TLR3 rs5743303 AA基因型相比，AT或TT基因型携带者的直肠癌发病风险明显增加(OR=1.43，95%CI 1.07~1.90)。而TLR4 rs1927914、TLR4 rs7869402、TLR5 rs1640816和TLR7 rs3853839遗传变异并不影响直肠癌的发病风险(P>0.05)。

表1　病例组与对照组基本资料比较[例(%)]

Tab.1　Comparison of basic data between patients and controls [n(%)]

项目	病例组(n=480)	对照组(n=480)	χ^2	P
性别			0.004	0.947
男	301(62.7)	302(62.9)		
女	179(37.3)	178(37.1)		
年龄(岁)			0.067	0.796
≤60	246(51.3)	250(52.1)		
>60	234(48.8)	230(47.9)		
吸烟			0.334	0.563
是	128(26.7)	136(28.3)		
否	352(73.3)	344(71.7)		
饮酒			0.234	0.629
是	158(32.9)	151(31.5)		
否	322(67.1)	329(68.5)		
累计吸烟量(包年)*			0.763	0.382
<30	59(12.3)	70(14.6)		
≥30	69(14.4)	66(13.8)		

*累计吸烟量(包年)=每日吸烟支数/20×吸烟年数

表2　Toll样受体遗传变异与直肠癌易感性的关系[例(%)]

Tab.2　Relations of TLR genetic variation to the susceptibility of rectal cancer [n(%)]

基因型	病例组(n=480)	对照组(n=480)	OR(95% CI)[a]	P
TLR3 rs5743303				
AA	335(69.8)	368(76.7)		
AT	128(26.7)	100(20.8)	1.41(1.04~1.91)	0.025
TT	17(3.5)	12(2.5)	1.57(0.74~3.33)	0.243
AT+TT	145(30.2)	112(23.3)	1.43(1.07~1.91)	0.015
TLR4 rs7869402				
CC	434(90.4)	414(86.3)		
CT	43(9.0)	62(12.9)	0.66(0.44~0.99)	0.049
TT	3(0.6)	4(0.8)	0.73(0.16~3.32)	0.688
CT+TT	46(9.6)	66(13.8)	0.66(0.45~0.99)	0.050
TLR4 rs1927914				
AA	178(37.1)	150(31.3)		
AG	220(45.8)	250(52.1)	0.74(0.56~0.98)	0.038
GG	82(17.1)	80(16.7)	0.86(0.59~1.26)	0.441
AG+GG	302(62.9)	330(68.8)	0.77(0.59~1.01)	0.057
TLR5 rs1640816				
GG	374(77.9)	395(82.3)		
AG	103(21.5)	82(17.1)	1.33(0.97~1.84)	0.080
AA	3(0.6)	3(0.6)	1.02(0.20~5.10)	0.980
AG+AA	106(22.1)	83(17.3)	1.32(0.96~1.82)	0.085
TLR7 rs3853839				
男				
GG	105(21.9)	112(23.3)		
GC	63(13.1)	61(12.7)	0.96(0.61~1.52)	0.879
CC	11(2.3)	5(1.0)	1.89(0.61~5.89)	0.270
GC+CC	74(15.4)	66(13.8)	1.03(0.67~1.60)	0.886
女				
G	238(49.6)	225(46.9)		
C	63(13.1)	77(16.0)	0.79(0.54~1.15)	0.217

[a]非条件logistic回归分析，以性别、年龄、吸烟或饮酒状况校正。

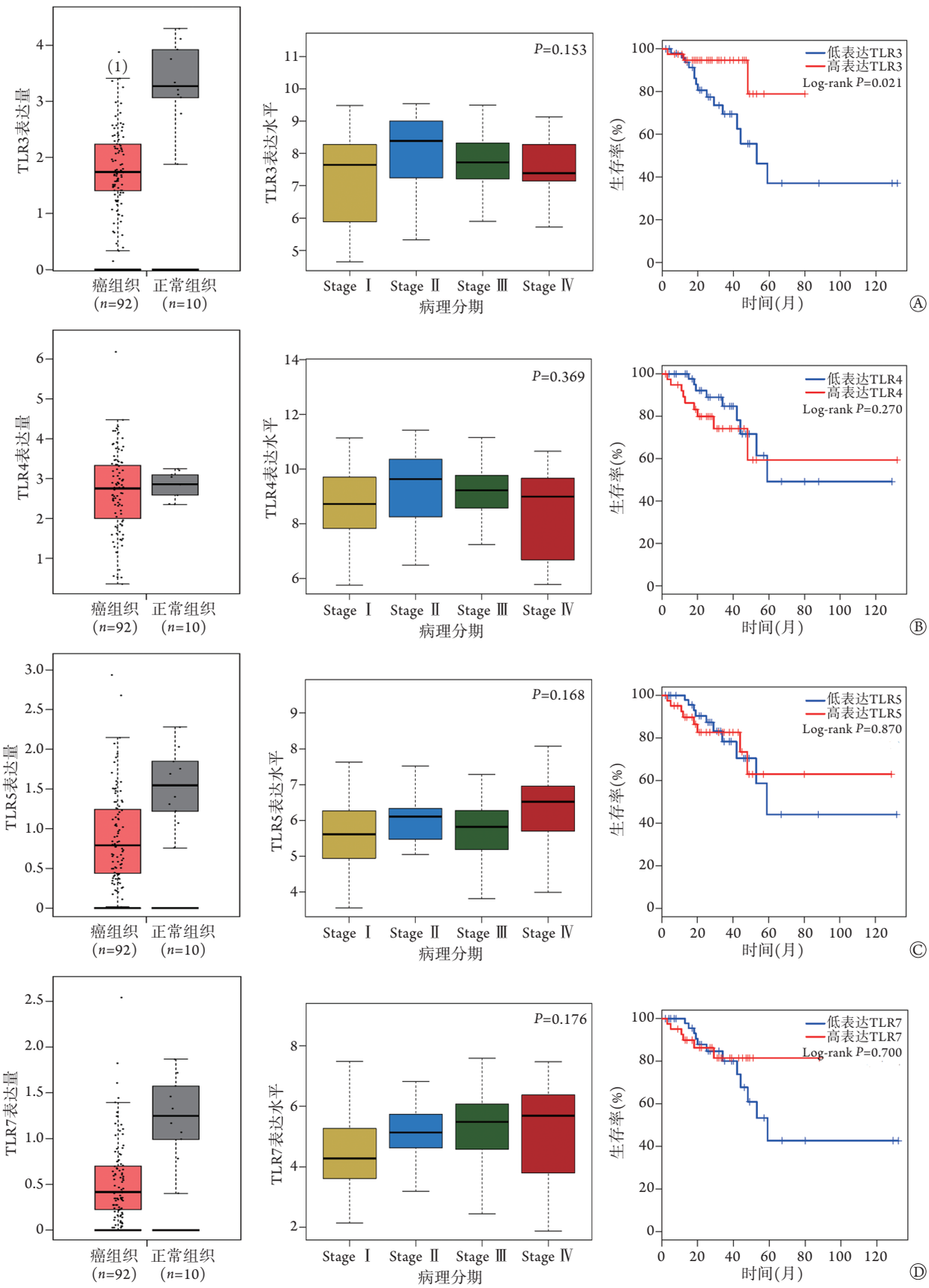

图1 TLRs在直肠癌中的表达及其与病理分期和预后的相关性分析

Fig.1 Expression of TLRs in rectal cancer tissues and its correlation with the pathological stage and prognosis of rectal cancer

A. TLR3；B. TLR4；C. TLR5；D. TLR7；与正常组织比较，(1)$P<0.05$。

TLR3 rs5743303遗传变异与直肠癌发病风险的分层分析结果如表3所示。性别分层结果显示，男性AT或TT基因型携带者的直肠癌发病风险是AA基因型携带者的1.47倍(P=0.041)，在女性中未见该变异影响直肠癌发病风险(P=0.202)。年龄分层结果显示，低年龄(≤60岁)AT或TT基因型携带者发生直肠癌的风险较高(OR=1.64，95% CI 1.08~2.47，P=0.019)，而在高龄(>60岁)者中差异无统计学意义(P=0.185)。吸烟和饮酒分层结果显示，与AA基因型携带者相比，携带AT或TT基因型的吸烟者(OR=2.42，95% CI 1.37~4.38)和饮酒者(OR=2.70，95% CI 1.52~4.80)发生直肠癌的风险较高(P=0.002，P=0.001)。

2.3　TLR3与免疫特征参数的关系　ESTIMATE算法分析结果显示，TLR3表达与直肠癌免疫评分呈正相关(r=0.23，P=0.038，图2A)。TIMER数据库分析结果显示，TLR3表达与$CD8^+$ T细胞的相关性最强(r=0.537，P<0.001)，与巨噬细胞无相关性(r=0.008，P=0.921，图2C)；体细胞TLR3拷贝数变异与B细胞、$CD8^+$ T细胞、$CD4^+$ T细胞、巨噬细胞、中性粒细胞和树突细胞的浸润程度呈弱相关或无相关性(P<0.05，图2B)。

表3　TLR3 rs5743303基因分型与直肠癌发病风险的分层分析

Tab.3　Stratified analysis of TLR3 rs5743303 genotypes with the risk of rectal cancer

项目	基因型(病例/对照)		(AT+TT)/AA OR(95% CI)[a]	P
	AA	AT+TT		
性别				
男	210/233	91/69	1.47(1.01~2.11)	0.041
女	125/135	54/43	1.37(0.84~2.21)	0.202
年龄(岁)				
≤60	172/197	74/53	1.64(1.08~2.47)	0.019
>60	163/171	71/59	1.32(0.87~2.00)	0.185
吸烟				
是	82/109	48/27	2.42(1.37~4.38)	0.002
否	253/259	97/85	1.19(0.85~1.67)	0.315
饮酒				
是	106/125	53/26	2.70(1.52~4.80)	0.001
否	229/243	93/86	1.16(0.82~1.64)	0.395

[a]非条件logistic回归分析，以性别、年龄、吸烟或饮酒状况校正。

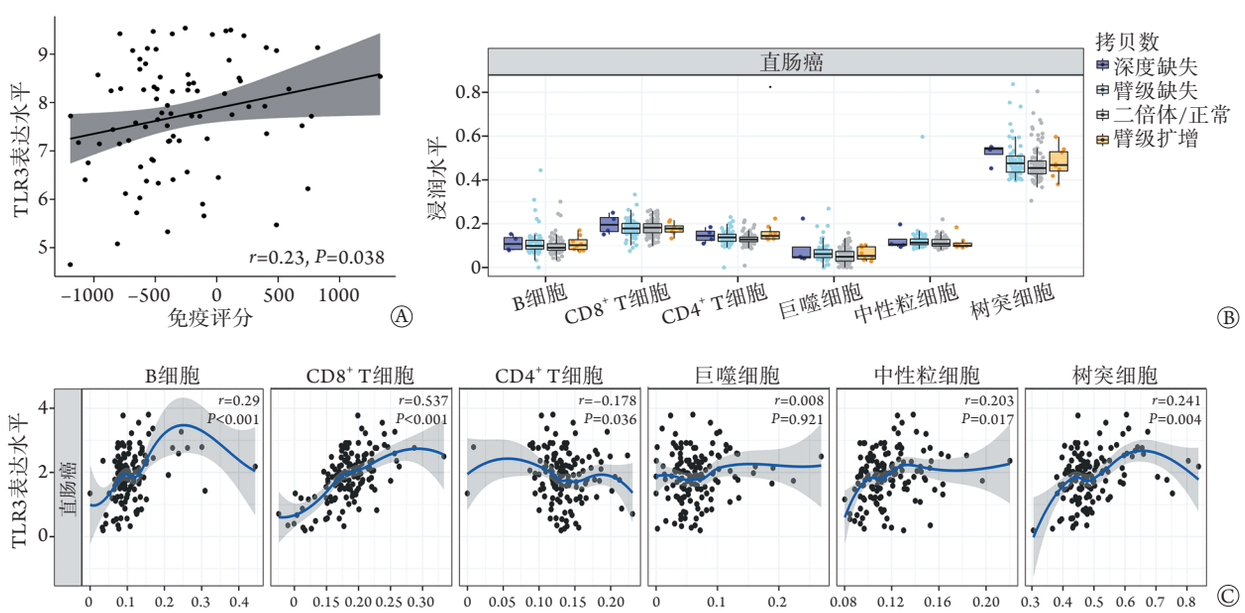

图2　TLR3与免疫特性参数的相关性分析

Fig.2　Correlation analysis between TLR3 and immune characteristic parameters

A. TLR3表达与免疫评分的相关性分析；B. 体细胞TLR3拷贝数变异与免疫浸润的相关性分析；C. TLR3表达与免疫浸润的相关性分析

2.4　直肠癌中TLR3的GO功能和GSVA富集分析　对TCGA数据库中直肠癌的RNA测序数据进行GO功能分析，结果显示，TLR3高表达主要与受体配体活性、抗原结合、免疫球蛋白受体结合、生长因子和细胞因子等相关基因功能的改变有关(图3A)。GSVA富集分析结果显示，TLR3相关通路主要富集在细胞凋亡、自然杀伤细胞介导的细胞毒性、磷脂代谢及自噬调节等与代谢或免疫相关的途径(图3B)。

3　讨　论

直肠癌是消化系统常见的恶性肿瘤之一。我国癌症统计数据显示，国人直肠癌发病率逐年上升且呈年轻化趋势[9]。尽管手术、靶向治疗及放化疗已在直肠癌的治疗中取得了满意的疗效，但患者5年总体生存率仍较低[10]。因此，对直肠癌发病及预后的影响因素进行探索，以发现直肠癌易感因素及预

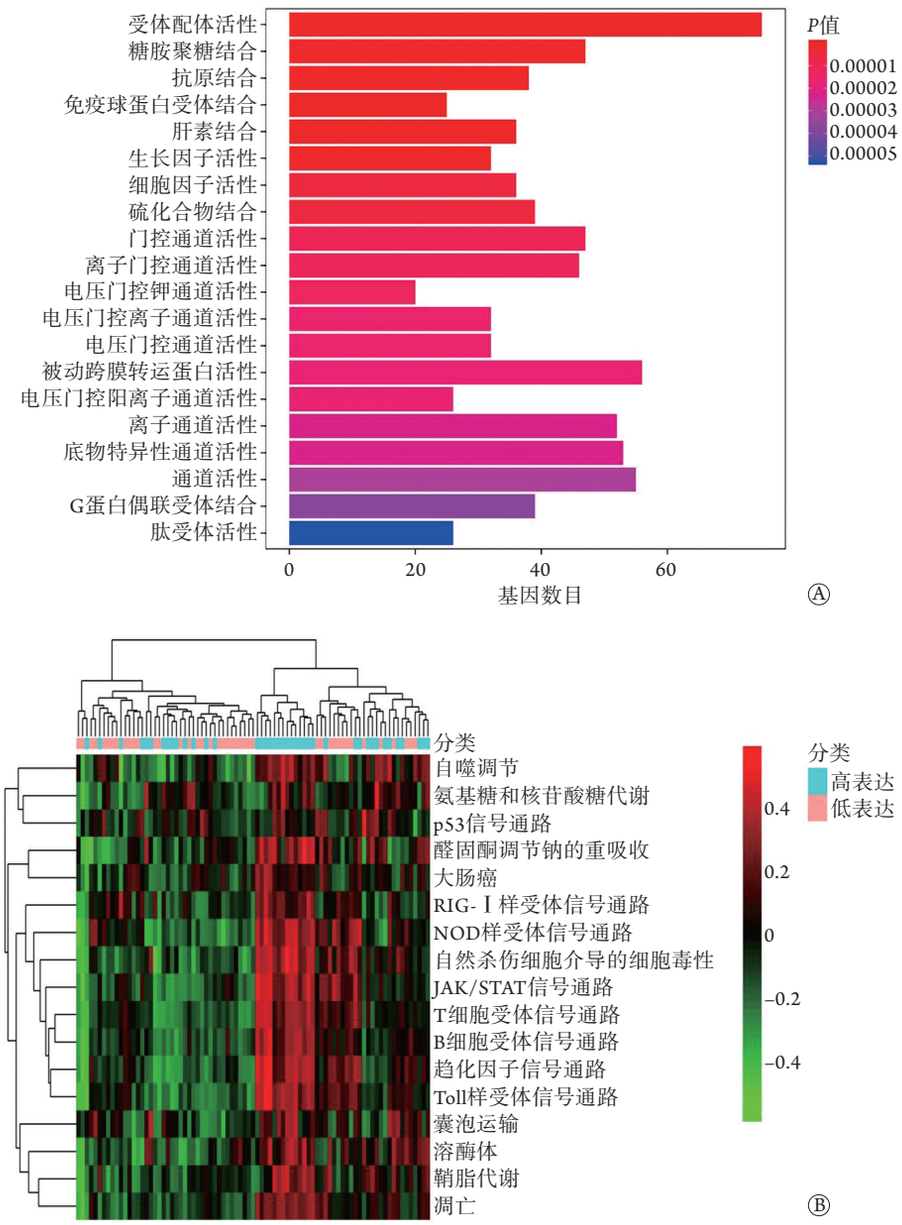

图3 TLR3表达对基因功能和信号通路的影响

Fig.3 Effect of TLR3 expression on gene function and signal pathway

A. GO功能分析；B. GSVA富集分析

后因素，有助于改善直肠癌患者的生存质量及预后情况。

TLRs是特殊的模式识别受体，可通过参与不同的病原相关分子模式(pathogen-associated molecular patterns，PAMP)和损伤相关分子模式(damage associated molecular patterns，DAMP)引起促炎因子、趋化因子和干扰素的释放而激活炎症通路，进而启动免疫反应[11]。TLRs可增加血管通透性，也可通过细胞毒性T细胞和自然杀伤细胞杀伤肿瘤细胞，从而发挥抑制肿瘤的作用[12]。在免疫治疗时代，肿瘤的发展受其内在特征及外在肿瘤微环境的共同影响。TLRs活化后经信号转导产生炎性因子并激活树突细胞[13]，而位于肿瘤微环境中的树突细胞可发挥抗原提呈作用，使自然杀伤细胞和T细胞活化，从而发挥细胞毒性作用，进而抑制肿瘤的发生发展[14]。

Bonnin等[15]发现，TLR3 mRNA及蛋白在肝癌组织中的表达均低于癌旁组织，且TLR3表达下调与肝癌患者生存率降低有关。对神经母细胞瘤和食管癌的研究发现，肿瘤实质中TLR3高表达与预后良好相关[16-17]，在非小细胞肺癌早期，TLR3高表达具有抑癌作用且与较高的总体生存率明显相关[18]。TLR3激动剂是一种强效免疫调节剂，可通过激活TLR3的表达来刺激抗原呈递细胞，从而活化肿瘤

特异性T细胞，以及将骨髓抑制细胞和肿瘤相关巨噬细胞的表型从免疫抑制转为免疫支持[19-21]。本研究生物信息学分析结果显示，先天免疫基因TLR3在直肠癌中低表达且与预后不良有关。肿瘤基因表达谱可以量化肿瘤微环境中的免疫活性[22]。本研究通过ESTIMATE和TIMER分析发现，TLR3与肿瘤微环境中的免疫细胞浸润具有一定的相关性，且GSVA富集分析结果也显示TLR3低表达可抑制自然杀伤细胞介导的细胞毒性通路，从而发挥促进肿瘤发展的作用。

TLRs基因中存在的SNP可能会损害其功能并破坏促炎与抗炎细胞因子之间的平衡，增加慢性炎症和癌症的发生风险[23-25]。TLR3 SNP影响癌症易感性，可作为评估癌症发生风险的潜在生物标志物[26]。与野生型相比，TLR3突变型与口腔癌和鼻咽癌的发病风险明显相关[27]。虽有研究表明TLR3基因多态性与乳腺癌的发病风险无关，但TLR3的表达与乳腺癌的侵袭性有关[28]。迄今为止，关于TLR3 SNP与直肠癌易感性关系的报道较少。本研究结果显示，TLR3低表达是直肠癌的危险因素，且TLR3 rs5743303的遗传变异与直肠癌发病风险增加明显相关，但直肠癌中TLR3 rs5743303遗传变异是否导致基因差异表达，尚需进一步研究验证。

既往研究发现，SNP可能通过破坏TLR4信号转导而增加慢性炎症和癌症的发生风险[29]，但尚未发现TLR4 rs1927914遗传变异与肺癌[30]、前列腺癌[31]、肝细胞癌[32]和胃癌[33]之间有任何明显关联。本研究亦未发现TLR4 rs1927914遗传变异与直肠癌发病风险有关。据报道，TLR5在多种癌症中表达并在癌变中起作用[34-36]。Chen等[37]发现，TLR5遗传变异与人类乳腺癌易感性存在密切关系。与其他TLR家族成员不同，TLR5在肠道上皮细胞中高度表达，并在宿主抵抗肠道细菌感染中发挥关键作用[38]。本研究结果显示，TLR5 rs1640816遗传变异与直肠癌发病风险无相关性。有研究发现，炎症性肠病患者与对照组TLR7 rs3853839等位基因频率无明显差异[39]。本研究亦未发现TLR7 rs3853839 G>C多态性与直肠癌发病风险有任何关联。

除遗传因素外，肿瘤的发生也与环境因素有关。流行病学研究表明，吸烟可能诱发表观遗传改变[40-41]，是直肠癌发病的适度危险因素[42-43]。本研究结果显示，至少携带一个TLR3 rs5743303 T等位基因的男性和低年龄者具有较高的直肠癌发病风险，且吸烟、饮酒可增加个体罹患直肠癌的风险，提示直肠癌的发生主要是环境因素和遗传因素共同作用的结果。

综上所述，本研究结果表明，TLR3低表达可能通过抑制免疫细胞活性及免疫相关途径促进直肠癌的发生与不良预后，且TLR3遗传变异可能增加个体罹患直肠癌的风险，但其具体作用机制尚需进一步研究证实。

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