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**Comprehensive analysis of** **distal-less homeobox family gene expression in colon cancer**

Chen YC *et al*. Role of DLX in colon cancer

Yong-Cheng Chen, Dong-Bing Li, Dong-Liang Wang, Hui Peng

**Yong-Cheng Chen,** Department of General Surgery (Endoscopic Surgery), The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

**Yong-Cheng Chen, Hui Peng,** Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

**Yong-Cheng Chen, Hui Peng,** Biomedical Innovation Center, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

**Dong-Bing Li, Dong-Liang Wang,** Department of Medicine, ChosenMed Technology (Beijing) Co., Ltd., Beijing 100176, China

**Hui Peng,** Department of General Surgery (Anorectal Surgery), The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

**Author contributions:** Chen YC and Peng H participated in study design, and data collection and analysis; Chen YC, Li DB, and Wang DL performed the data analysis; Chen YC and Peng H drafted the manuscript; Chen YC and Peng H revised the manuscript; All authors read and approved the final manuscript.

**Corresponding author: Hui Peng, MD, Chief Doctor,** Department of General Surgery (Anorectal Surgery), The Sixth Affiliated Hospital, Sun Yat-sen University, No. 26 Yuancun Erheng Road, Tianhe District, Guangzhou 510655, Guangdong Province, China. phui@mail.sysu.edu.cn

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**Abstract**

BACKGROUND

The distal-less homeobox (*DLX*) gene family plays an important role in the development of several tumors. However, the expression pattern, prognostic and diagnostic value, possible regulatory mechanisms, and the relationship between *DLX* family genes and immune infiltration in colon cancer have not been systematically reported.

AIM

We aimed to comprehensively analyze the biological role of the *DLX* gene family in the pathogenesis of colon cancer.

METHODS

Colon cancer tissue and normal colon tissue samples were collected from the Cancer Genome Atlas and Gene Expression Omnibus databases. Wilcoxon rank sum test and *t*-test were used to assess *DLX* gene family expression between colon cancer tissue and unpaired normal colon tissue. cBioPortal was used to analyze *DLX* gene family variants. R software was used to analyze *DLX* gene expression in colon cancer and the relationship between *DLX* gene family expression and clinical features and correlation heat map. The survival package and Cox regression module were used to assess the prognostic value of the *DLX* gene family. The pROC package was used to analyze the diagnostic value of the *DLX* gene family. R software was used to analyze the possible regulatory mechanisms of *DLX* gene family members and related genes. The GSVA package was used to analyze the relationship between the *DLX* gene family and immune infiltration. The ggplot2, the survminer package, and the clusterProfiler package were used for visualization.

RESULTS

*DLX1/2/3/4/5* were significantly aberrantly expressed in colon cancer patients. The expression of *DLX* genes were associated with M stage, pathologic stage, primary therapy outcome, residual tumor, lymphatic invasion, T stage, N stage, age, perineural invasion, and history of colon polyps. *DLX5* was independently correlated with the prognosis of colon cancer in multivariate analysis. *DLX1/2/3/4/5/6* were involved in the development and progression of colon cancer by participating in immune infiltration and associated pathways, including the Hippo signaling pathway, the Wnt signaling pathway, several signaling pathways regulating the pluripotency of stem cells, and *Staphylococcus aureus* infection.

CONCLUSION

The results of this study suggest a possible role for the *DLX* gene family as potential diagnostic or prognostic biomarkers and therapeutic targets in colon cancer.

**Key Words:** Colon cancer; The Cancer Genome Atlas; Distal-less homeobox genes; Prognosis; Immune infiltration

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**Core Tip:** The distal-less homeobox (*DLX*) gene family plays an important role in the pathogenesis of several tumors. However, the expression pattern, prognostic and diagnostic value, possible regulatory mechanisms, and the relationship between *DLX* family genes and immune infiltration in colon cancer have not been systematically reported. In this study, we aimed to investigate the expression level, clinical significance, and relationship between *DLX* genes and immune infiltration in colon cancer to establish an adequate scientific basis for clinical decision making and risk management. The *DLX* gene family holds promise as a potential diagnostic or prognostic biomarker and therapeutic target for colon cancer.

**INTRODUCTION**

Colon cancer comprises a widely study group of tumors whose incidence is on the rise. Approximately 10% of all cancer deaths are caused by colon cancer and related complications[1]. Colon adenocarcinoma (COAD) is the most common, accounting for 98% of colon cancer cases[2]. Colon cancer has a high recurrence rate after treatment, with 42% of patients recurring within 5 y and a median time from recurrence to death of 12 mo[3]. Unfortunately, about 20% of colon cancer patients are diagnosed with stage IV each year[4]. Therefore, exploring novel molecular markers is of great clinical significance to improve the diagnosis and treatment of colon cancer.

The distal-less homeobox (*DLX*) gene is a homolog of *Drosophila* distal-less and consists of 6 members, including *DLX1, DLX2, DLX3, DLX4, DLX5*, and *DLX6*[5]. *DLX1* can be used to identify prostate cancer for early diagnosis[6]. Overexpression of *DLX2* has been associated with poor prognosis in hepatocellular carcinoma (HCC)[7]. High expression of *DLX2* has been shown to be a poor prognostic marker for patients with glioblastoma multiforme[8]. *DLX3* has been demonstrated as a key regulator of the STAT3 signaling network that maintains skin homeostasis[9]. *DLX4* can also be used as a prognostic marker for HCC[10]. *DLX5* has been shown to be a potential diagnostic biomarker and therapeutic target for oral squamous cell carcinoma (OSCC)[11]. *DLX6* has been shown to promote cell proliferation and survival in OSCC[12]. To our knowledge, no studies have systematically assessed the role of the *DLX* gene family in colon cancer using bioinformatics methods. In this study, we aimed to investigate the expression level, clinical significance, and relationship between *DLX* family genes and immune infiltration in colon cancer to establish an adequate scientific basis for clinical decision making and risk management.

**MATERIALS AND METHODS**

***CBioPortal analysis***

The cBio Cancer Genomics Portal (cBioPortal) (http://cbioportal.org) was used to study mutations in *DLX* genes in colon cancer[13]. Queries for visualization and analysis were performed using the following entries: (1) Cancer type: COAD; (2) 2 selected studies: COAD (CaseCCC, PNAS 2015), colon cancer (CPTAC-2 Prospective, Cell 2019); (3) Molecular profile: Mutations and copy number alterations (CNAs); (4) Selection of patients/case sets: All samples (139); and (5) Input genes: *DLX1*(ENSG00000144355), *DLX2*(ENSG00000115844), *DLX3*(ENSG00000064195), *DLX4*(ENSG00000108813), *DLX5*(ENSG00000105880), and *DLX6*(ENSG00000006377). After submission of queries, accessions were made including origin studies, mutation profiles, mutation number, overall survival (OS) status, OS (months), disease-free status, and disease-free period (months) tracks.

***Dysregulation of DLX genes in colon cancer***

R software (version 3.6.3) was used for statistical analysis and visualization[14,15]. The R packages used included ggplot2 (version 3.3.3) for visualization. UCSC XENA (https://xenabrowser.net/datapages/) RNAseq data were uniformly processed by the Toil process into TPM (transcripts per million reads) format for the Cancer Genome Atlas (TCGA) and GTEx[16]. Data for colon cancer were extracted from the TCGA and corresponding normal tissue data were extracted from GTEx. RNAseq data were in TPM format and log2 transformed for expression comparisons between samples. The data filtering condition was set to retain paired samples.

***Correlation heat map***

Correlation between every 2 genes of the *DLX* family was assessed using a Pearson’s correlation coefficient. The R package used was mainly ggplot2 (version 3.3.3). The filter condition was set to remove data from the normal/control groups (of note, not every item had a normal/control group).

***Association of DLX gene expression with clinical features of TCGA-colon cancer***

The R package used was the basic R package[17]. Grouping was based on the median.

***Survival analysis***

The survminer package (version 0.4.9) was used for visualizing survival data, and the survival package (version 3.2-10) allowed statistical analysis of survival data. Subgroups included 0-50 and 50-100. The prognosis types were OS, progression-free interval (PFS), and disease specific survival (DSS). Supplementary data were prognostic data from the reference literature[18]. The filter condition was set to remove data from the normal/control groups (of note, not every item had a normal/control group) and keep the data for clinical information.

***Univariate and multivariate Cox regression analysis***

The R package used was the survivor package (version 3.2-10). Statistical analysis was performed using the Cox regression module. Prognosis types were OS, PFS, and DSS, and included variables were *DLX1, DLX2, DLX3, DLX4, DLX5*, and *DLX6*. Supplementary data were prognostic data from the reference literature[18]. The filter condition was set to remove data from the normal/control groups (of note, not every item had a normal/control group) and keep the data for clinical information.

***ROC curve analysis***

Two R packages were used: the pROC package (for analysis) and ggplot2 package (version 3.3.3). Clinical variables were “tumor” and “normal”. UCSC XENA (https://xenabrowser.net/datapages/) RNAseq data were uniformly processed by the Toil process into TPM format for TCGA and GTEx[16]. Data for colon cancer were extracted from TCGA and corresponding normal tissue data were extracted from GTEx. The RNAseq data were in TPM format and log2 transformed for expression comparison between samples. Data were not filtered. The horizontal coordinate was the false positive rate and the vertical coordinate was the true positive rate.

***Correlation analysis for genes associated with DLX genes***

The R package used was the stat package (version 3.6.3) (base package). The TCGA colon cancer project provided the RNAseq data in level 3 HTSeq-FPKM format. The TPM format was converted to FPKM, and log2 transformation was applied to the transformed data. The control/normal groups were removed from the results (of note, not all projects had control/normal groups).

***Functional enrichment analysis of genes associated with DLX genes***

The R packages used were mainly ggplot2 package (version 3.3.3) and clusterProfiler package (version 3.14.3).

***Correlation between the expression of DLX genes in colon cancer and immune cells***

The R package used was the GSVA package (version 1.34.0)[19]. For immune infiltration, the GSVA package had a built-in algorithm, ssGSEA. Immune cells included were activated dendritic cells (aDCs), B-cells, CD8 T-cells, cytotoxic cells, dendritic cells (DCs), eosinophils, immature DCs (iDCs), macrophages, mast cells, neutrophils, natural killer (NK) CD56bright cells, NK CD56dim cells, NK cells, plasmacytoid DCs (pDCs), T-cells, T helper (Th) cells, T central memory cells, T effector memory (Tem) cells, T follicular helper (TFH) cells, T gamma delta (Tgd) cells, Th1 cells, Th17 cells, Th2 cells, and regulatory T (Treg) cells[20]. The data filtering condition was set to remove the control/normal group (of note, not all projects had control/normal groups). Markers for 24 immune cells were obtained from the reference literature[21].

***Validation of DLX gene expression***

To further verify the accuracy of the TCGA database, we downloaded colon cancer samples from the Gene Expression Omnibus database for analysis. The 30 colon cancer tissue samples and 30 normal colon tissue samples contained in GSE74062 were used for *DLX* gene expression analysis.

***Statistical analysis***

All statistical analyses were performed using R software (v.3.6.3). The Wilcoxon rank sum test, chi-square test, and Fisher exact test were used to analyze the relationship between clinical characteristics and *DLX* genes. *P* values less than 0.05 were considered statistically significant.

**RESULTS**

***DLX gene alterations and mRNA expression in colon cancer***

The cBioPortal online tool was used to analyze the expression of *DLX* family genes in colon cancer patients. Alterations in the expression of *DLX* genes in colon cancer ranged from 0.7% to 3% (Figure 1). The mutation data, CNA data, and deep deletion from the 2 studies are depicted in Figure 2. The analysis of *DLX* gene expression was performed based on 41 colon cancer tissue samples and 41 paired samples of normal colon tissue (Figure 3). The results showed that the expression level of *DLX1* in colon cancer was significantly lower than that in normal colon tissue (0.199 ± 0.026 *vs* 0.867 ± 0.031; *P* < 0.001). The expression level of *DLX2* in colon cancer was significantly lower than that in normal colon tissue (0.129 ± 0.020 *vs* 0.211 ± 0.011; *P* = 0.0074). The expression level of *DLX3* in colon cancer was significantly higher than that in normal colon tissue (0.593 ± 0.052 *vs* 0.171 ± 0.008; *P* < 0.001). The expression level of *DLX4* in colon cancer was significantly higher than in normal colon tissue (0.635 ± 0.027 *vs* 0.229 ± 0.009; *P* < 0.001). The expression level of *DLX5* in colon cancer was significantly lower than that in normal colon tissue (0.416 ± 0.036 *vs* 0.463 ± 0.022; *P* < 0.001). There was no significant difference in *DLX6* expression in colon cancer compared to normal colon tissue (0.229 ± 0.014 *vs* 0.449 ± 0.037; *P* = 0.554). We examined the correlation between *DLX* genes using Pearson correlation analysis. There was no significant correlation between *DLX1* and *DLX3*, *DLX1* and *DLX6*; there was a significant positive correlation between other *DLX* genes (Figure 4).

***Relationship between DLX gene expression and clinical characteristics and prognosis of colon cancer patients***

Clinical characteristics data and gene expression data for 478 colon cancer samples were downloaded from the TCGA database (Supplementary Table 1). *DLX2* expression was associated with M stage (*P* = 0.005), pathologic stage (*P* = 0.014), primary therapy outcome (*P* = 0.036), residual tumor (*P* = 0.002), and lymphatic invasion (*P* = 0.013). *DLX3* expression was associated with N stage (*P* < 0.001), M stage (*P* < 0.001), pathologic stage (*P* < 0.001), height (*P* = 0.045), and residual tumor (*P* < 0.001). *DLX5* expression was associated with T stage (*P* < 0.001), N stage (*P* < 0.001), M stage (*P* = 0.005), pathologic stage (*P* < 0.001), primary therapy outcome (*P* = 0.005), age (*P* < 0.001), perineural invasion (*P* = 0.023), lymphatic invasion (*P* < 0.001), and history of colon polyps (*P* = 0.009). However, the expression of *DLX1, DLX4*, and *DLX6* did not significantly correlate with any clinical characteristic of colon cancer patients.

A low expression of *DLX1* was associated with PFS (*P* = 0.013); a low expression of *DLX2* was associated with OS (*P* = 0.006), PFS (*P* = 0.003), and DSS (*P* = 0.007); a high expression of *DLX3* was associated with OS (*P* = 0.010), PFS (*P* = 0.004), and DSS (*P* = 0.007); a high expression of *DLX4* was associated with OS (*P* = 0.030) and PFS (*P* = 0.023); a low expression of *DLX5* was associated with poor OS (*P* = 0.048), PFS (*P* = 0.002), and DSS (*P* = 0.007). However, a high expression of *DLX6* was not significantly associated with prognosis in colon cancer (Figure 5).

Univariate Cox regression analysis for OS showed that *DLX2* (*P* = 0.007), *DLX3* (*P* = 0.011), *DLX4* (*P* = 0.031), and *DLX5* (*P* = 0.049) were associated with OS, and *DLX1* (*P* = 0.014), *DLX2* (*P* = 0.003), *DLX3* (*P* = 0.004), *DLX4* (*P* = 0.024), and *DLX5* (*P* = 0.002) were associated with PFS. *DLX2* (*P* = 0.008), *DLX3* (*P* = 0.008), and *DLX5* (*P* = 0.009) were associated with DSS. *DLX5* was independently correlated with PFS (*P* = 0.012) and DSS (*P* = 0.035) in multivariate analysis (Table 1).

*DLX1* had some accuracy in diagnosing normal and tumor outcomes [area under curve (AUC) = 0.893; 95%CI: 0.867-0.920]. *DLX2* also had some accuracy in diagnosing normal and tumor outcomes (AUC = 0.731; 95%CI: 0.691-0.771), while *DLX3* had a lower accuracy in diagnosing these outcomes (AUC = 0.561; 95%CI: 0.512-0.611). *DLX4* also had some accuracy in diagnosing normal and tumor outcomes (AUC = 0.834; 95%CI: 0.802-0.867), while *DLX5* had low accuracy in diagnosing these outcomes (AUC = 0.590; 95%CI: 0.546-0.635). Lastly, *DLX6* had poor accuracy in diagnosing normal and tumor outcomes (AUC = 0.486; 95%CI: 0.439-0.534) (Figure 6).

***The function of genes associated with DLX genes***

The top 10 significantly associated genes for each *DLX* gene are shown in the single gene co-expression heat map (Figure 7). Genes significantly associated with *DLX1* included *DLX2, KLF14, CHRND, KCNN1, IGDCC3, ARHGAP36, NCAN, TFAP2B, CNPY1*, and *CACNG7*. Genes significantly associated with *DLX2* included *DLX1, CNPY1, CHRND, NEUROD1, IGDCC3, TNFRSF19, KLF14, NELL2, HS3ST4*, and *SLC38A8*. Genes significantly associated with *DLX3* included *NOTUM, NKD1, APCDD1, ADAMTSL2, MYH7B, PRR9, LRRC43, CAB39L, ABCC2*, and *DLX4*. Genes significantly associated with *DLX4* included *DLX3, TTLL4, DNMT3B, CDK5R1, IGF2BP1, STK36, UNK, AMER3, PHF12*, and *WNT3*. Genes significantly associated with *DLX5* included *DYNC1I1, DLX6, RASL11B, ID4, SP7, AMBN, KRT31, MYL3, VENTX*, and *ISM1*. Genes significantly associated with *DLX6* included *DLX5, TRIM71, SH3GL2, SLC46A1, DYNC1I1, PGBD5, GAL, COCH, AXIN2*, and *CKB*. The top 30 genes significantly associated with each *DLX* gene (147 in total) were analyzed for Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment (Supplementary Table 2). The top biological processes included pattern specification, regionalization, ossification, connective tissue development, cell fate commitment, hippocampus development, biomineral tissue development, biomineralization, skeletal system morphogenesis, and odontogenesis. The significantly related molecular functions included DNA-binding transcription activator activity, RNA polymerase II-specificity, fibroblast growth factor receptor binding, DNA-binding transcription activator activity (Figure 8 and Supplementary Table 3). The significantly related pathways included the Hippo signaling pathway, the Wnt signaling pathway, and signaling pathways regulating the pluripotency of stem cells and *Staphylococcus aureus* infection (Figure 9 and Supplementary Table 3).

***Correlation of DLX gene expression and immune cells in colon cancer***

There was a correlation between *DLX* gene expression and immune cells in colon cancer (Figure 10). *DLX1* gene expression positively correlated with some tumor-infiltrating immune cells (TIICs), including aDCs, cytotoxic cells, DCs, eosinophils, iDCs, macrophages, mast cells, neutrophils, NK CD56dim cells, NK cells, Tem cells, TFH cells, Tgd cells, Th1 cells, and Treg cells; *DLX1* expression negatively correlated with Th17 cells. *DLX2* gene expression positively correlated with mast cells and TFH cells and negatively correlated with pDCs and Th17 cells. *DLX3* gene expression negatively correlated with some TIICs, including aDCs, CD8 T-cells, cytotoxic cells, DCs, macrophages, neutrophils, T-cells, Th cells, Th1 cells, Th2 cells, and Treg cells. *DLX4* gene expression positively correlated with NK cells and negatively correlated with some TIICs, including cytotoxic cells, DCs, macrophages, pDCs, Th1 cells, and Th2 cells. *DLX5* gene expression positively correlated with some TIICs, including B-cells, CD8 T-cells, DCs, iDCs, macrophages, mast cells, neutrophils, NK cells, pDCs, Tem cells, TFH cells, Tgd cells, and Treg cells; *DLX5* expression negatively correlated with Th17 cells and Th2 cells. *DLX6* gene expression negatively correlated with some TIICs, including aDCs, cytotoxic cells, DCs, macrophages, neutrophils, NK CD56dim cells, T-cells, Tem cells, and Th1 cells.

***DLX genes were aberrantly expressed in colon cancer tissue***

Compared to normal colon, *DLX1* (*P* = 7.6e-08), *DLX2* (*P* = 5.7e-08), *DLX4* (*P* = 0.00013), and *DLX5* (*P* = 0.0084) were aberrantly expressed in colon cancer tissue. However, *DLX3* and *DLX6* were not aberrantly expressed in colon cancer (Figure 11).

**DISCUSSION**

*DLX1* has been shown to be significantly upregulated in prostate cancer tissues and cells[22]. *DLX2* is known to be significantly upregulated in HCC tissues and cell lines[7,23], and its expression in gastric cancer has been shown to significantly correlated with tumor size, depth of infiltration, lymph node metastasis, and tumor-lymph node metastasis stage[24]. *DLX4* has been demonstrated to be upregulated in nasopharyngeal carcinoma (NPC) cell lines[25], and its expression was shown to be elevated in HCC and correlated significantly with tumor size, histopathological classification, and serum alpha-fetoprotein[10]. *DLX5* has been shown to be upregulated in OSCC tissues and cell lines, and has been associated with advanced TNM staging, lymph node metastasis, poor cell differentiation, and tumor location[11]. *DLX6* has been shown to be upregulated in oral cancer and has been associated with advanced tumor stage and poor prognosis[12]. In this study, *DLX1/2/3/4/5* were aberrantly expressed in colon cancer tissue samples. The expression of *DLX* family genes was associated with M stage, pathologic stage, primary therapy outcome, residual tumor, lymphatic invasion, T stage, N stage, age, perineural invasion, and history of colon polyps. In the multivariate analysis, *DLX5* was independently related to PFS and OS. In diagnosing the outcome of normal and tumor tissues, *DXL1/2/4* had some accuracy.

MiR-129-5p has been shown to impede the biological function of cancer cells by inhibiting *DLX1* expression[26]. *DLX1*, a key target of *FOXM1*, has been shown to promote ovarian cancer aggressiveness by enhancing transforming growth factor (TGF)-β/SMAD4 signaling[27]. Circ\_*HIPK3* has been demonstrated to promote HCC progression by mediating the miR-582-3p/*DLX2* pathway[23]. In tumor cells, *DLX2/3/4* can be involved in the control of fenretinide (4HPR)-mediated apoptosis[28]. *DLX3* has been shown to be downregulated by miR-133[29]. The homology domain protein *DLX4* has been shown to promote NPC progression through the upregulation of YB-1[25]. *DLX5* regulation of *CCND1* affected the progression of OSCC[11]. *DLX5* has been shown topromote osteosarcoma progression through activation of the NOTCH signaling pathway[30]. *DLX6* has been demonstrated to regulate OSCC cell proliferation through the *EGFR-CCND1* axis[12]. In this study, the *DLX* gene family is suggested to be involved in the development and progression of colon cancer by participating in several pathways, including breast cancer, gastric cancer, the Hippo signaling pathway, the Wnt signaling pathway, and signaling pathways regulating the pluripotency of stem cells, basal cell carcinoma, melanoma, and *Staphylococcus aureus* infection. Dlx-2 is involved in TGF-β- and Wnt-induced inhibition of mitochondria by epithelial-mesenchymal transition, glycolytic conversion, and Snail activation[31]. However, the specific mechanisms by which the *DLX* gene family mediates the pathways involved in the development of colon cancer need to be further investigated.

Immune-related mechanisms play an important role in the development of colon cancer, and immunotherapeutic strategies are considered a promising direction for the treatment of this disease[32]. Another important aspect of the current study was that the expression of the *DLX* gene family correlated with different levels of immune infiltration. Here, the expression levels of *DLX* family genes were negatively correlated with some TIICs, and positively correlated with other TIICs. The *DLX* gene family plays an important role in the recruitment and regulation of immune infiltrating cells in colon cancer.

The present study has several limitations. Firstly, colon cancer shows strong heterogeneity, and the mRNA expression levels in the TCGA database are the average mRNA expression levels for all cell types within various colon tumors. Single-cell sequencing is needed to further elucidate the role of *DLX* genes in colon cancer and its subtypes. Secondly, our study findings are not confirmed by biological or molecular experiments.

**CONCLUSION**

*DLX1/2/3/4/5* were significantly aberrantly expressed in colon cancer tissue samples. *DLX 2/3/5* were associated with M stage, pathologic stage, primary therapy outcome, residual tumor, lymphatic invasion, T stage, N stage, age, perineural invasion, and history of colon polyps. *DLX5* was independently correlated with the prognosis of colon cancer in multivariate analysis. *DLX1/2/4* had some accuracy in diagnosing normal and tumor conditions. The *DLX* gene family may be involved in the development and progression of colon cancer by participating in immune infiltration and pathways, including the Hippo signaling pathway, the Wnt signaling pathway, and signaling pathways regulating the pluripotency of stem cells and *Staphylococcus aureus* infection. The results of this study suggest a role for *DLX* family genes as a potential diagnostic or prognostic biomarkers and therapeutic targets in colon cancer.

**ARTICLE HIGHLIGHTS**

***Research background***

The distal-less homeobox (*DLX*) gene family plays an important role in several tumors. However, the role of *DLX* gene family in colon cancer is not yet clear.

***Research motivation***

The aim of this study was to investigate the role of the *DLX* gene family in colon cancer and to establish a sound scientific basis for clinical decision making and risk management.

***Research objectives***

In this study, we aimed to comprehensively analyze the biological role of the *DLX* gene family in colon cancer.

***Research methods***

Colon cancer and normal colon tissue samples were collected from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus databases. We used Wilcoxon rank sum test and t-test to assess *DLX* gene family expression between colon cancer tissue samples and unpaired normal colon tissue samples, cBioPortal to analyze *DLX* gene family variants, R software (version 3.6.3) to analyze *DLX* gene expression in colon cancer and the relationship between *DLX* gene family expression and clinical features and correlation heat map, the survival package [version 3.2-10] and Cox regression module to assess the prognostic value of the *DLX* gene family, the pROC package [version 1.17.0.1] to analyze the diagnostic value of the *DLX* gene family, R software (version 3.6.3) to analyze the possible regulatory mechanisms of *DLX* gene family members and related genes, the GSVA package [version 1.34.0] to analyze the relationship between the *DLX* gene family and immune infiltration, and the ggplot2 [version 3.3.3], the survminer package [version 0.4.9], and the clusterProfiler package [version 3.14.3] for visualization.

***Research results***

Expression levels of *DLX1/2/3/4/5* were significantly abnormal in tissue from patients with colon cancer. *DLX* gene family expression in colon cancer was significantly associated with clinical characteristics, including M stage, pathological stage, primary treatment outcome, residual tumor, lymphatic invasion, T stage, N stage, age, peripheral invasion, and history of colonic polyps. Results of the multivariate Cox analysis showed *DLX5* to be an independent prognostic factor in patients with colon cancer. *DLX1/2/3/4/5/6* may be involved in the development and progression of colon cancer through mediation of multiple pathways, including the Hippo signaling pathway, the Wnt signaling pathway, and signaling pathways regulating the pluripotency of stem cells. *DLX1/2/3/4/5/6* are associated with immune infiltration.

***Research conclusions***

*DLX* family genes may function as potential diagnostic or prognostic biomarkers and therapeutic targets for colon cancer.

***Research perspectives***

It may be possible to use *DLX* family genes as a diagnostic or prognostic biomarkers or therapeutic targets for colon cancer.

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**Footnotes**

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**Clinical trial registration statement:** This study did not involve a clinical trial registration statement.

**Informed consent statement:** The data that support the findings of this study are publicly available. The current study does not require signed informed consent documents.

**Conflict-of-interest statement:** All the authors report having no relevant conflicts of interest for this article.

**Data sharing statement:** All data and material are public.

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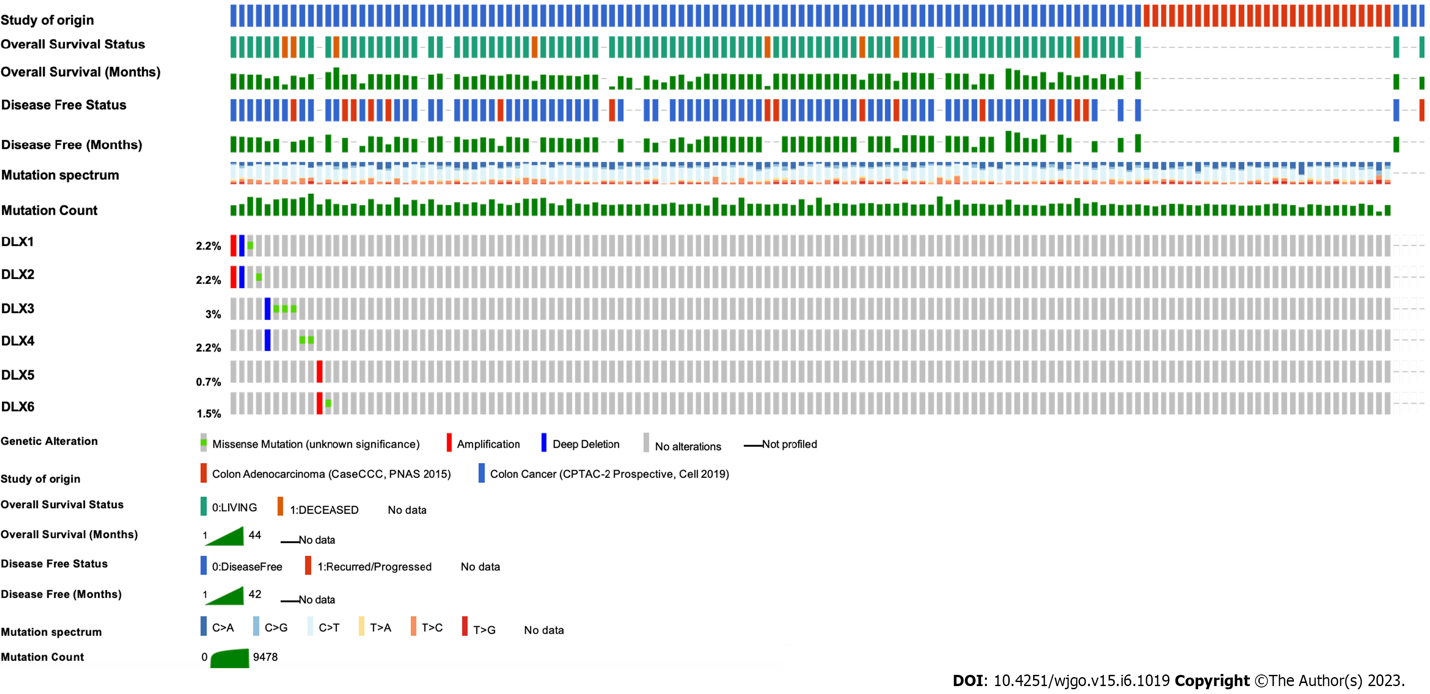
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**Figure Legends**



**Figure 1 mRNA expression of distal-less homeobox genes in colon adenocarcinoma in cBioPortal (RNA Seq V2 RSEM).** DLX: Distal-less homeobox.

图表

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**Figure 2 Percentage of distal-less homeobox genes in colon adenocarcinoma cases calculated using the cancer type summary in cBioPortal.**

图表, 箱线图

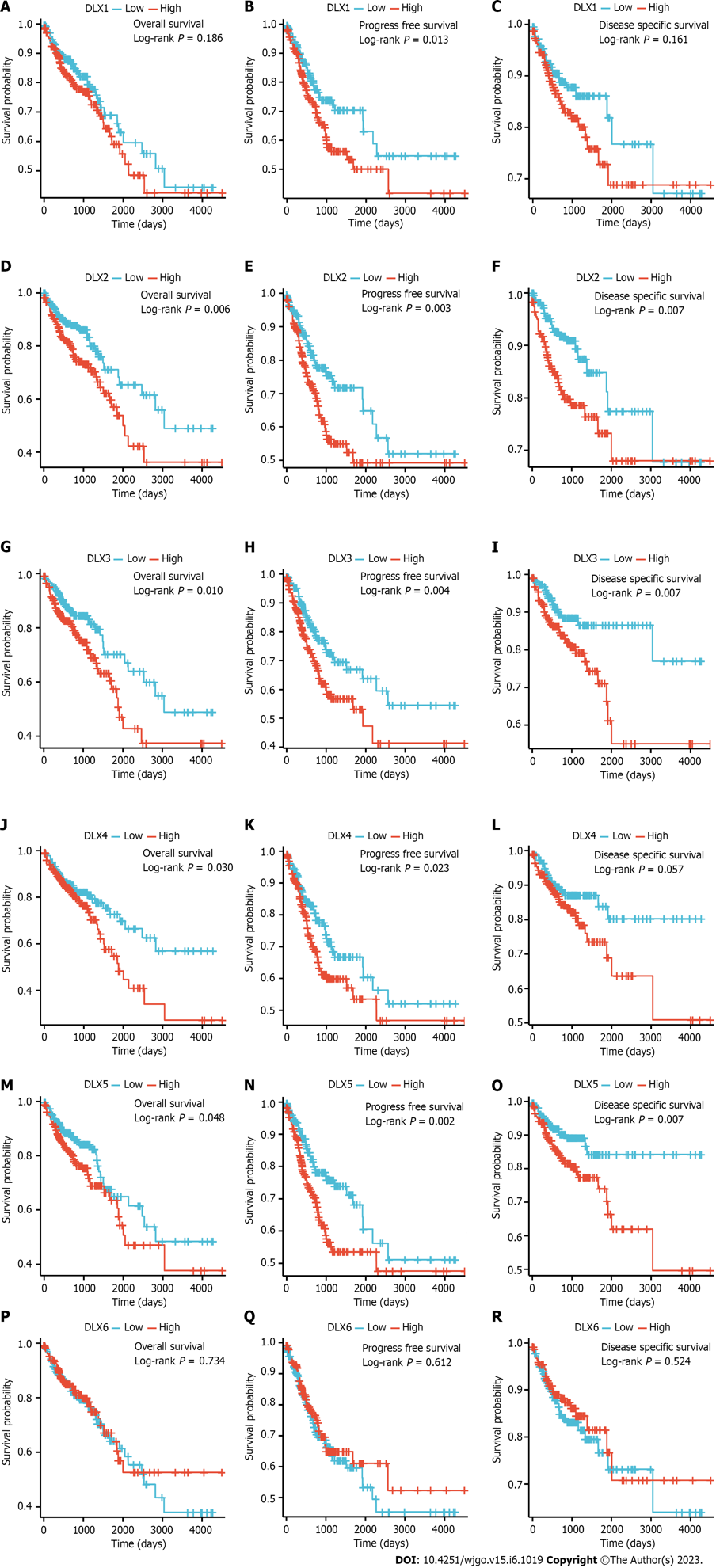
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**Figure 3 mRNA levels of distal-less homeobox genes between colon adenocarcinoma tissue and unpaired normal stomach tissue in the Cancer Genome Atlas.** b*P* < 0.01; c*P* < 0.001. *DLX*: Distal-less homeobox.

表格

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**Figure 4 Correlation between every two genes of** **distal-less homeobox genes in colon adenocarcinoma.** b*P* < 0.01; c*P* < 0.001. *DLX*: Distal-less homeobox.

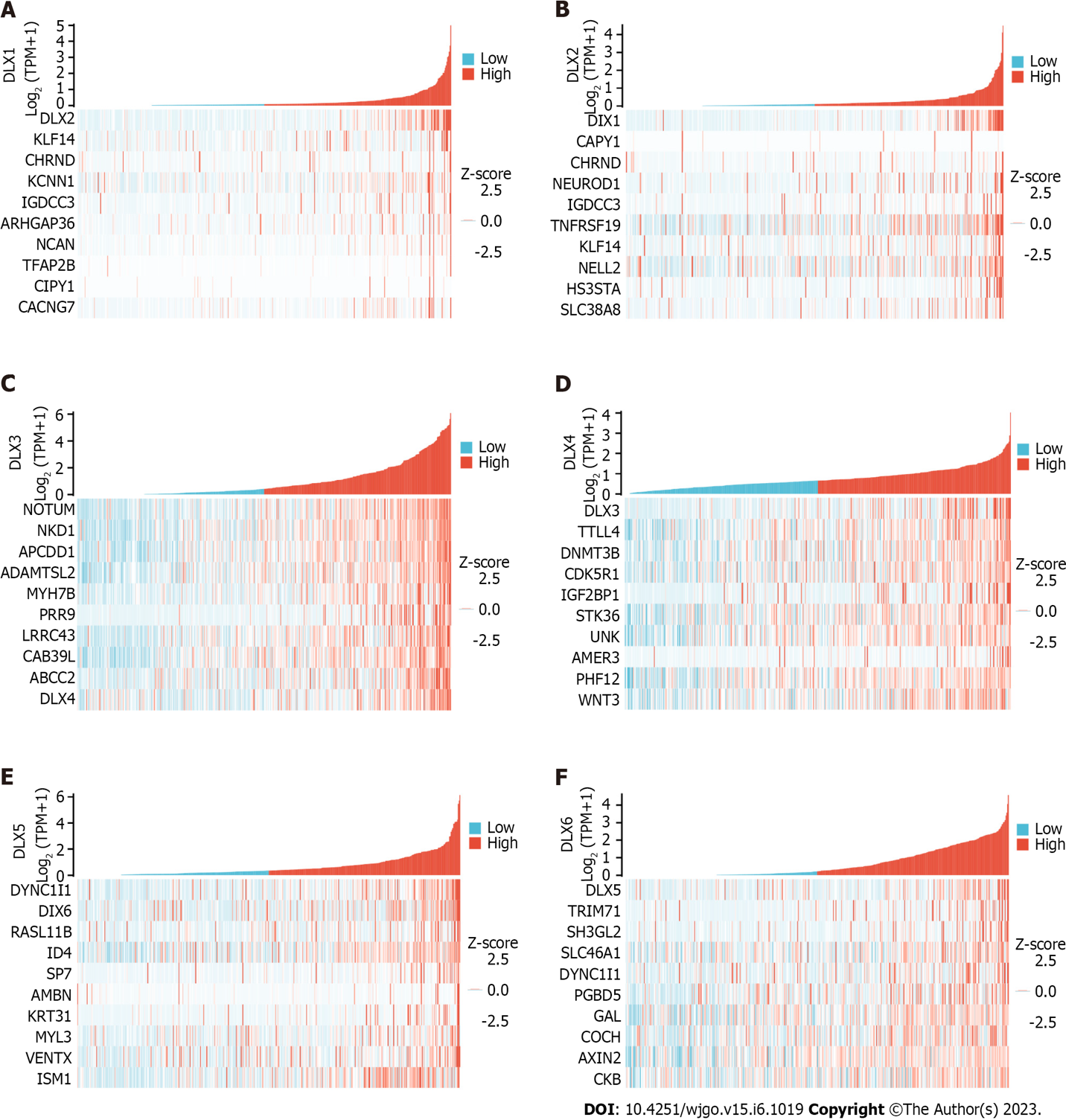


**Figure 5 Survival analysis results for distal-less homeobox genes.** A: Overall survival (OS) of distal-less homeobox (*DLX*)1; B: Progression-free survival (PFS) of *DLX1*; C: Disease specific survival (DSS) of *DLX1*; D: OS of *DLX2*; E: PFS of *DLX2*; F: DSS of *DLX2*; G: OS of *DLX3*; H: PFS of *DLX3*; I: DSS of *DLX3*; J: OS of *DLX4*; K: PFS of *DLX4*; L: DSS of *DLX4*; M: OS of *DLX5*; N: PFS of *DLX5*; O: DSS of *DLX5*; P: OS of *DLX6*; Q: PFS of *DLX6*; R: DSS of *DLX6*. *DLX*: Distal-less homeobox.

图表

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**Figure 6 Receiver operating characteristic curves of distal-less homeobox genes in colon adenocarcinoma and normal colon tissues.** The area under the receiver operating characteristic curve is between 0.5 and 1. The closer the area under the curve (AUC) is to 1, the better the diagnosis. the AUC is between 0.5 and 0.7 with low accuracy, the AUC is between 0.7 and 0.9 with some accuracy, and the AUC is above 0.9 with high accuracy. AUC: Area under the curve; *DLX*: Distal-less homeobox; FPR: False positive rate; TPR: True positive rate.



**Figure 7 Heatmap plot of top 10 correlated genes to distal-less homeobox genes.** A: Distal-less homeobox (*DLX*)1; B: *DLX2*; C: *DLX3*; D: *DLX4*; E: *DLX5*; F: *DLX6*. *DLX*: Distal-less homeobox.

图表, 条形图

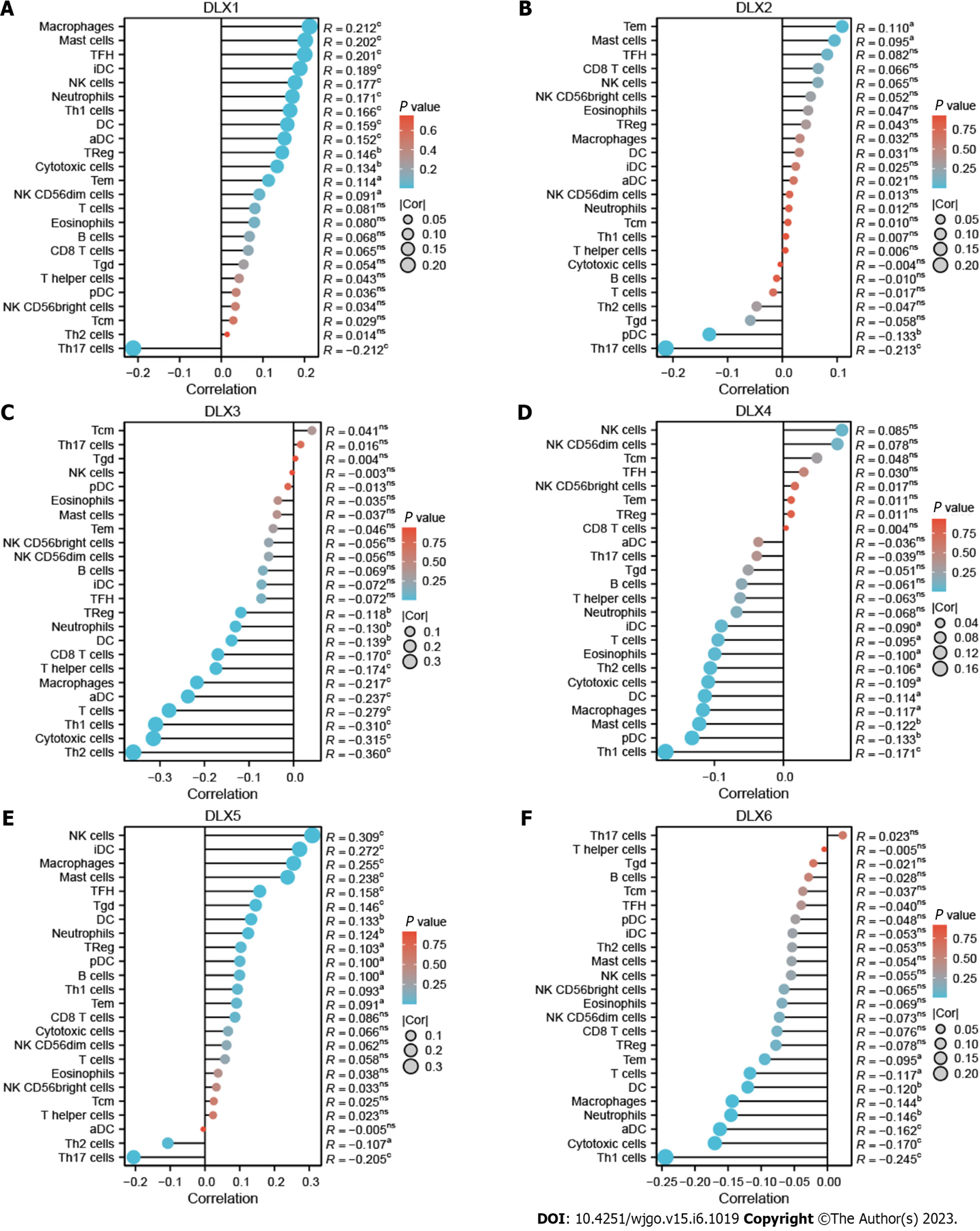
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**Figure 8** **Gene Ontology analysis of genes associated with distal-less homeobox genes.** BP: Biological process; MF: Molecular function.

图示

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**Figure 9** **Kyoto Encyclopedia of Genes and Genomes analysis of genes associated with distal-less homeobox genes.** KEGG: Kyoto Encyclopedia of Genes and Genomes.



**Figure 10 Correlation between the expression of each** **distal-less homeobox gene and the 24 tumor-infiltrating immune cells of colon adenocarcinoma (lollipop plot).** In the color bar, the darker the color, the smaller the *P*-value, indicating a higher statistical significance. The bubble size represents the correlation value, the larger the bubble, the larger the correlation value. A: Correlation between distal-less homeobox (*DLX1*) expression and immune infiltration; B: Correlation between *DLX2* expression and immune infiltration; C: Correlation between *DLX3* expression and immune infiltration; D: Correlation between *DLX4* expression and immune infiltration; E: Correlation between *DLX5* expression and immune infiltration; F: Correlation between *DLX6* expression and immune infiltration. a*P* < 0.05; b*P* < 0.01; c*P* < 0.001. aDC: Activated dendritic cell; DC: Dendritic cell; *DLX*: Distal-less homeobox; iDC: Immature dendritic cell; NK: Natural killer; Tcm: T central memory; Tem: T effector memory; TFH: T follicular helper; Tgd: T gamma delta; Th: T helper.

图示, 示意图

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**Figure 11 Differential expression of** **distal-less homeobox genes in colon adenocarcinoma and normal colon tissues (GSE74062).** A: Distal-less homeobox (*DLX*)1; B: *DLX2*; C: *DLX4*; D: *DLX5*. b*P* < 0.01; c*P* < 0.001. *DLX*: Distal-less homeobox.

**Table 1 Univariate and multivariate Cox regression analyses with** **distal-less homeobox genes and prognosis of colon adenocarcinoma patients**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Survival** | **Characteristics** | **Total, *n*** | **Univariate analysis** | | **Multivariate analysis** | |
| **HR (95%CI)** | ***P* value** | **HR (95%CI)** | ***P* value** |
| Overall | *DLX1* (low *vs* high) | 477 | 1.299 (0.880-1.917) | 0.186 |  |  |
| *DLX2* (low *vs* high) | 477 | 1.736 (1.167-2.584) | 0.007 | 1.502 (0.988-2.285) | 0.057 |
| *DLX3* (low *vs* high) | 477 | 1.668 (1.123-2.476) | 0.011 | 1.374 (0.900-2.099) | 0.142 |
| *DLX4* (low *vs* high) | 477 | 1.538 (1.039-2.276) | 0.031 | 1.197 (0.783-1.830) | 0.405 |
| *DLX5* (low *vs* high) | 477 | 1.485 (1.001-2.202) | 0.049 | 1.334 (0.893-1.993) | 0.159 |
| *DLX6* (low *vs* high) | 477 | 0.935 (0.634-1.379) | 0.734 |  |  |
| Progression-free | *DLX1* (low *vs* high) | 477 | 1.557 (1.094-2.214) | 0.014 | 1.316 (0.901-1.921) | 0.155 |
| *DLX2* (low *vs* high) | 477 | 1.715 (1.201-2.449) | 0.003 | 1.317 (0.883-1.964) | 0.178 |
| *DLX3* (low *vs* high) | 477 | 1.670 (1.174-2.376) | 0.004 | 1.365 (0.937-1.990) | 0.105 |
| *DLX4* (low *vs* high) | 477 | 1.497 (1.054-2.125) | 0.024 | 1.181 (0.813-1.715) | 0.382 |
| *DLX5* (low *vs* high) | 477 | 1.742 (1.217-2.492) | 0.002 | 1.588 (1.105-2.283) | 0.012 |
| *DLX6* (low *vs* high) | 477 | 0.914 (0.646-1.294) | 0.613 |  |  |
| Disease specific | *DLX1* (low *vs* high) | 461 | 1.426 (0.865-2.349) | 0.164 |  |  |
| *DLX2* (low *vs* high) | 461 | 2.014 (1.202-3.376) | 0.008 | 1.666 (0.971-2.857) | 0.064 |
| *DLX3* (low *vs* high) | 461 | 2.007 (1.202-3.349) | 0.008 | 1.570 (0.909-2.713) | 0.106 |
| *DLX4* (low *vs* high) | 461 | 1.617 (0.981-2.664) | 0.059 | 1.179 (0.692-2.011) | 0.545 |
| *DLX5* (low *vs* high) | 461 | 2.011 (1.193-3.390) | 0.009 | 1.765 (1.039-2.998) | 0.035 |
| *DLX6* (low *vs* high) | 461 | 0.852 (0.520-1.395) | 0.524 |  |  |

CI: Confidence interval; *DLX*: Distal-less homeobox; HR: Hazard ratio.



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