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Characterization of the prognostic values of the *NDRG* family in gastric cancer

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Abstract

Background: The N-myc downstream-regulated gene (*NDRG*) family, *NDRG1-4*, has been involved in a wide spectrum of biological functions in multiple cancers. However, their prognostic values remain sparse in gastric cancer (GC). Therefore, it is crucial to systematically investigate the prognostic values of the *NDRG* family in GC.

Methods: The prognostic values of the *NDRG* family were evaluated by Kaplan–Meier Plotter and SurvExpress. The mRNA of the *NDRG* family was investigated in The Cancer Genome Atlas (TCGA). Transcription factors (TFs) and miRNAs associated with the *NDRG* family were predicted by NetworkAnalysis. The prognostic values of DNA methylation levels were analyzed by MethSurv. The correlation between immune cells and the *NDRG* family was evaluated by the Tumor Immune Estimation Resource (TIMER) database.

Results: High levels of mRNA expression of *NDRG2* and *NDRG3* were associated with a favorable prognosis in all GCs. In *HER2*$^{-}$ GC, *NDRG1* was significantly associated with a poor prognosis of GC [hazard ratio (HR) = 1.65, 95% confidence interval (CI) = 1.16–2.33, $p = 0.0046$]. In *HER2*$^{+}$ GC, *NDRG4* showed a poor prognosis (HR = 1.4, 95% CI: 1.06–1.85, $p = 0.017$). *NDRG4* was an independent prognostic factor in recurrence-free survival by TCGA cohort. The low-risk *NDRG*-signature group displayed a significantly favorable survival outcome than the high-risk group (HR = 1.76, 95% CI: 1.2–2.59, $p = 0.00385$). The phosphorylated protein NDRG1 (NDRG1_pT346) displayed a favorable overall survival and was significantly associated with *HER2* and phosphorylated *HER2*. Epidermis development was the top biological process (BP) for coexpressed genes associated with *NDRG1* and *NDRG4*, while mitotic nuclear division and mitotic cell processes were the top BPs for *NDRG2* and *NDRG3*, respectively. Overall, 6 CpGs of *NDRG1*, 4 CpGs of *NDRG2*, 3 CpGs of *NDRG3* and 24 CpGs of *NDRG4* were associated with significant prognosis. CD4^{+} T-cells showed the highest correlation with *NDRG4* (correlation = 0.341, $p = 2.14e^{-11}$). Furthermore, BCL6 in follicular helper T-cells (Tfh) cells showed the highest association with *NDRG4* (correlation = 0.438, $p = 00e^{+}00$).

Conclusions: This study analyzed the multilevel prognostic values and biological roles of the *NDRG* family in GC.

Keywords: gastric cancer, *HER2*, methylation, *NDRG* family, prognosis, TCGA

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Introduction

Gastric cancer (GC) is one of the leading death-causing, malignant diseases in eastern Asia.[1–3] Although improved dietary habits, solid diagnostic screening systems, multiprincipled therapeutic regimes and updated surgical techniques have reduced both the incidence and mortality rates of GC,[4–6] the prognosis of GC remains

unsatisfactory.[3] Thus, identification of reliable biomarkers for the prognostic prediction of GC could facilitate individualized clinical management.

The N-myc downstream-regulated gene (*NDRG*) family consists of four members, *NDRG1*, *NDRG2*, *NDRG3* and *NDRG4*, located on chromosomes 8q24.3, 14q11.2, 20q11.21-23 and 16q21-q22.1 respectively.[7–8] Although the four members share 57–65% of amino acid sequences with an alpha/beta hydrolase-fold and an NDR region, they lack catalytic motifs and therefore do not have a hydrolase function.[8] *NDRG1–4* have been found to be widely expressed in human organs and multiple biological functions have been recently discovered.[9] The molecular functions of the *NDRG* family cover a wide spectrum of biological processes, including cell development and differentiation, stress responses and proliferation, tumor progression and metastasis.[7,10–19] *NDRG1* has been implicated in embryonic placentation, organ development and cellular skeleton modification,[7,10,11] and is induced by hypoxia and DNA damage.[12] Global gene expression analysis of breast epithelial cells indicates that *NDRG1* is closely associated with cellular vesicle transport and regulation of membrane proteins, such as low-density lipoprotein and E-cadherin endosomal trafficking.[13–15] The prognostic values of *NDRG1* in solid tumors have been intensively investigated. In esophageal cancer, low *NDRG1* mRNA expression indicates a worse prognosis.[20] It is also negatively correlated with tumor progression and metastasis in colorectal, breast and prostate cancers,[12,21] while associated with an unfavorable prognosis for hepatocellular carcinoma.[22]

NDRG2, regulated by maturation-associated stimuli, is strongly expressed in dendritic cells[16] and is able to maintain activated leukocyte cell adhesion during the entire differentiation progress of dendritic cells.[17] *NDRG2* expression is found significantly reduced in pancreatic, breast and hepatocellular carcinomas compared with normal counterparts.[23–25] Specifically, reduced expression of *NDRG2* is correlated with aggressive tumor behavior, higher recurrence and distant metastasis ratio in hepatocellular carcinoma.[24] Of note, *NDRG2* expression has been found to be negatively associated with a worse prognosis in GC and prostate cancer.[26,27]

NDRG3 promotes angiogenesis and cell growth and is also involved in the lactate-dependent hypoxia signaling pathway.[18] High levels of *NDRG3* are associated with shorter overall survival (OS) and relapse-free survival (RFS) in advanced prostate cancer.[27]

NDRG4 is exclusively expressed in the central nervous system and heart in the embryonic stage, highlighting its essential role of regulating growth and proliferation.[19] *NDRG4* is reduced in both mRNA and protein expression in colorectal cancer tissues and functionally suppressed in tumor invasion and cell proliferation,[28] and is associated with a favorable survival.[29]

Collectively, the prognostic values of the *NDRG* family have been noticed in various types of cancers. However, the whole picture of the prognostic value of the entire *NDRG* family remains poorly investigated in GC. Hereby, based on updated public resources and integrative bioinformatics analysis, the prognostic value of the *NDRG* family was comprehensively assessed.

Methods

Survival analysis in Kaplan–Meier plotter

The prognostic values of mRNA expression of each *NDRG* family member to OS were analyzed based on Kaplan–Meier (KM) plotter, a website database based on resources from the Gene Expression Omnibus, including GSE14210, GSE15459, GSE22377, GSE29272, GSE51105 and GSE62254. In fact, GSE62254 was excluded from the total sample survival analysis given its markedly different clinical and genomic data, as suggested by KM plotter. Survival data in each subgroup, including pathological stage, Lauren classification, histological differentiation and human epidermal growth factor receptor 2 (*HER2*) status, were collected respectively. All four members of the *NDRG* family were analyzed with various parameters in KM plotter (http://kmplot.com/analysis/index.php?p=service&cancer=gastric).[30] The best cutoff values were determined by algorithms embedded in KM plotter.[30] The final prognostic KM plots were presented with a hazard ratio (HR), 95% confidence interval (CI) and log-rank p value. A p value <0.05 was considered statistically significant.

Prognosis analysis of NDRG *signature* via *SurvExpress platform*

The prognostic value of the *NDRG* family signature was analyzed *via* SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp), which is a platform for integrating public available resources for survival assessment.[31] The Stomach Adenocarcinoma (STAD) data of TCGA were selected as the input resource ($n = 352$). High/low-risk groups were determined by the algorithm of the prognostic risk score. Risk score = $\exp_{\text{mRNA of NDRG1}} \times \text{beta}_{\text{mRNA of NDRG1}} + \exp_{\text{mRNA of NDRG2}} \times \text{beta}_{\text{mRNA of NDRG2}} + \exp_{\text{mRNA of NDRG3}} \times \text{beta}_{\text{mRNA of NDRG3}} + \exp_{\text{mRNA of NDRG4}} \times \text{beta}_{\text{mRNA of NDRG4}}$, where 'exp' indicates the standardized mRNA expression of each selected gene, and 'beta' was obtained from the Cox multivariate regression analysis.[31] Moreover, the receiver operating characteristics (ROC) curve was used to evaluate the survival curves of the *NDRG* signature over different event times using the R package, survivalROC.[31]

Analysis of the mRNA expression of the NDRG *family in TCGA*

The mRNA expression of the *NDRG* family was explored in the pathological stage-specific pattern (one-way analysis of variance, violin plots) and among the tumor and normal tissues in the STAD data of TCGA in the Gene Expression Profiling Interactive Analysis platform (GEPIA; http://gepia.cancer-pku.cn/index.html). This web-based tool was established for customized investigation of genomic functionalities based on the resources provided by TCGA and the genotype-tissue expression (GTEx) projects.[32] Furthermore, the mRNA expression of the *NDRG* family, along with other clinic-pathological data, was downloaded from the Xena system (University of California, Santa Cruz, CA, USA), for statistical analysis.[33]

Protein expression of NDRG1–4 *in the Human Protein Atlas*

Protein expression of *NDRG1–4* in both GC and normal tissues was retrieved from the Human Protein Atlas (www.proteinatlas.org).[34]

Analysis of the reverse-phase protein array data of The Cancer Proteome Atlas

The Cancer Proteome Atlas (TCPA) dataset (http://tcpaportal.org/tcpa/index.html) mainly provides a comprehensive resource for the assessment, visualization and analysis of cancer proteomic data based on TCGA tumor tissue sample sets. Reverse-phase protein array (RPPA) data are used as a high-throughput antibody-dependent experimental procedure with increased quality and robust quantification.[35] The phosphorylated *NDRG1* (*NDRG1*_pT364) and epidermal growth factor receptor (EGFR), *HER2*, as well as corresponding phosphorylated data (EGFR_pY1068, EGFR_pY1173, HER2_pY1248) were extracted for correlation.[35]

Prognostic value of NDRG1_*pT346* via *TRGAted platform*

The TRGAted platform (https://nborcherding.shinyapps.io/TRGAted/), is a web tool for survival analysis based on RPPA data retrieved from TCGA.[36] Given only *NDRG1*_pT346 was available in the RPPA of STAD, we only accessed the prognostic value of *NDRG1*_pT346, including OS and disease-free survival (DFS), between high and low expression groups. The optimal cutoff was determined based on the surv-cutpoint function in the survminer package *via* TRGAted.[36] HR was determined by the Cox proportional hazard regression model.[36]

Gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the coexpressed genes of NDRG1–4

The genomic alterations of *NDRG1–4* were analyzed by cBioPortal, an integrative analytic platform of TCGA.[37,38] The coexpressed genes of *NDRG1–4* with a Pearson correlation ($\geqslant 0.3$ or $\leqslant -0.3$) were subject to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID).[39–41] The cutoff value was a false discover rate (FDR) <0.05 for significant GO and KEGG data.

Prediction of transcription factors and miRNAs for NDRG1–4

Potential transcription factors (TFs) and miRNAs of *NDRG1–4* were predicted by NetworkAnalysis (http://www.networkanalyst.ca).[42,43] The prediction of TFs for *NDRG1–4* was based on the ENCODE database with ChIP-seq data. Only the data with a peak intensity signal value <500

and a potential score value <1 was screened for further analysis. The miRNA-gene interaction data were retrieved from TarBase and miRTarBase *via* the NetworkAnalysis platform.

DNA methylation data of NDRG1–4 *in MethSurv*

The DNA methylation of *NDRG1–4* in TCGA was analyzed by MethSurv (https://biit.cs.ut.ee/methsurv/).[44] The prognostic values and expression levels of CpG methylation in *NDRG1–4* were explored.

Tumor-immune infiltrating cells associated with the NDRG *family* via *the Tumor Immune Estimation Resource database*

Correlations between all tumor-immune infiltrating cells (TIICs) and the *NDRG* family were analyzed *via* the Tumor Immune Estimation Resource (TIMER) platform (https://cistrome.shinyapps.io/timer/), a web tool for gene-specific correlational analysis with TIICs. TIICs included B-cells, $CD4^+$T-cells, $CD8^+$T-cells, dendritic cells, macrophages and neutrophils.[45] Tumor purity was used for the correction of Spearman-based correlation analysis.[45] Moreover, TIICs with the highest correlation to the *NDRG* family were selected for further subtype-based biomarker analysis.[46,47] Corresponding markers included TBX21 (T-bet), STAT4, STAT1, IFNG (interferon gamma) and TNF (tumor necrosis factor) for T helper (Th)1 cells; BCL6, interleukin (IL)21 for Tfh; GATA3, STAT6, STAT5A and IL13 for Th2; FOXP3, CCR8, STAT5B and TGFB1 (transforming growth factor beta) for T regulatory (Treg) cells; PDCD1 (programmed cell death 1), CTLA4, LAG3, HAVCR2 (TIM-3), GZMB for T-cell exhaustion; STAT3 and IL17A for Th17 cells.[46,47]

Statistically analysis

SPSS 17.0 (Chicago, IL, USA) and Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and illustration. A student's *t* test and Pearson correlation test were used for comparison between groups and correlation analysis. Cox regression was used for univariate and multivariate survival analysis. A p value <0.05 was considered significant in all circumstances.

Results

Prognostic values of NDRG *members in the whole group of patients with GC*

The prognostic values of *NDRG* mRNA expression in the whole group of patients with GC from KM plotter were collected [Figure 1(a–e)]. *NDRG2* and *NDRG3* were significantly associated with a better OS prognosis [Figure 1(a, c and d), HR = 0.64, 95% CI: 0.52–0.80, $p < 0.0001$ and HR = 0.78, 95% CI: 0.63–0.96, $p = 0.021$] while *NDRG1* and *NDRG4* showed a modest association with a worse prognosis for the OS [Figure 1(a, b and e), HR = 1.21, 95% CI: 0.98–1.49, $p = 0.072$ and HR = 1.2, 95% CI: 099–1.45, $p = 0.068$].

Prognostic values of NDRG *members in* $HER2^{+/-}$ *GC patients*

Next, the prognostic values of *NDRG* family members in $HER2^{+/-}$ GC were assessed [Figure 2(a–i)]. Of note, high mRNA expression of *NDRG1* was correlated with a favorable prognosis of $HER2^+$ GC patients, but was not statistically significant [Figure 2(a, i), HR = 0.78, 95% CI = 0.58–1.06, $p = 0.11$]. Of note, *NDRG1* displayed a significantly unfavorable prognosis in $HER2^-$ GC [Figure 2(e, i), HR = 1.65, 95% CI = 1.16–2.33, $p = 0.0046$]. Similarly, *NDRG4* showed an inverse prognosis between $HER2^{+/-}$ groups. However, the prognostic value of *NDRG4* in $HER2^-$ was not significant. In addition, *NDRG2* showed a favorable outcome in both $HER2^{+/-}$ groups [Figure 2(b, f)]. *NDRG3* only showed a favorable outcome in the $HER2^+$ group [Figure 2(c)].

Prognostic values of the NDRG *family with different clinicopathological features*

In the Lauren classification, a high mRNA expression of *NDRG2* was correlated with a worse prognosis in mixed types [Figure 3(a), HR = 5.07, 95% CI: 1.1–23.28, $p = 0.021$]. *NDRG3* [Figure 3(B), HR = 0.58, 95% CI: 0.34–0.99, $p = 0.045$] was correlated with a favorable prognosis in diffuse types. High mRNA expression of *NDRG4* was correlated with a worse prognosis in intestinal types [Figure 3(c), HR = 2.02, 95% CI: 1.33–3.06, $p = 0.00079$]. In histological differentiation, high mRNA expression of *NDRG1* was correlated with a worse prognosis in poor differentiation

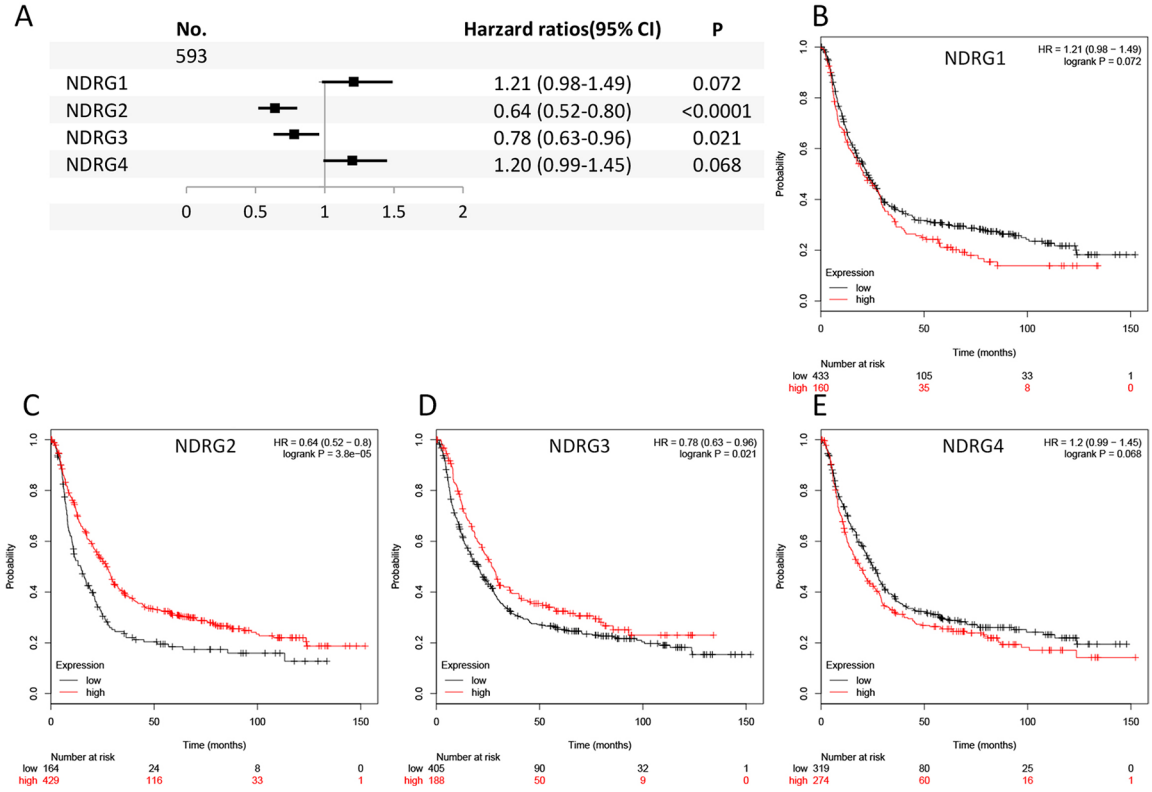

Figure 1. The prognostic value of the *NDRG* family mRNA expression in the KM plotter database. (a). Forest plot of the prognostic HRs of *NDRG* family members in total GC patients. (b–e). Survival curves of *NDRG1* (Affymetrix IDs: 200632_s_at), *NDRG2* (Affymetrix IDs: 206453_s_at), *NDRG3* (Affymetrix IDs: 217286_s_at), *NDRG4* (Affymetrix IDs: 209159_s_at) for all GC patients ($n = 593$).
Red: high expression level; black: low expression level.
GC, gastric cancer; HR: hazard ratio; KM, Kaplan–Meier.

types [Figure 3(d), HR = 1.84, 95% CI: 1.08–3.15, $p = 0.023$], and high mRNA expressions of *NDRG2* and *NDRG4* were correlated with worse prognosis in well differentiated types [Figure 3(e), HR = 3.32, 95% CI: 1.36–8.15, $p = 0.0056$; Figure 3(f), HR = 11.61, 95% CI: 1.55–87.17, $p = 0.0027$]. The rest of the *NDRG* members showed no significant prognostic correlation in both Lauren and histological subtypes.

We next evaluated the prognostic values of *NDRG* family members on distant metastasis status, lymph node status and pathological stages. High mRNA expression of *NDRG2* was correlated with better prognosis in the distant metastasis negative group [Figure 3(g), HR = 0.51, 95% CI: 0.32–0.81, $p = 0.0041$]. Furthermore, high mRNA expressions of *NDRG1* and *NDRG2* were found to be correlated with better prognosis in lymph node-negative and positive subgroups respectively [Figure 3(h), HR = 0.25, 95% CI: 0.08–0.73, $p = 0.0069$; HR = 0.65, 95% CI: 0.42–1.00, $p = 0.05$]. In pathological stages, high mRNA expression of *NDRG4* was correlated with a worse prognosis in stage II and III while *NDRG3* was correlated with a better prognosis [Figure 3(i), HR = 0.48, 95% CI: 0.25–0.89, $p = 0.018$].

NDRG4 *was validated as an independent prognostic factor*

The prognostic values of the *NDRG* family had been studied in KM plotter. Furthermore, they were further validated in the TCGA database (STAD). The Cox regression was analyzed for both univariate and multivariate process, including sex, age, TNM stage and mRNA expression of the *NDRG* family (Table 1 and 2). The results indicated that only *NDRG*4 was determined as an independent prognostic factor for GC in recurrence-free survival results (HR = 1.247, 95% CI: 1.057–1.470, $p = 0.009$; Table 2).

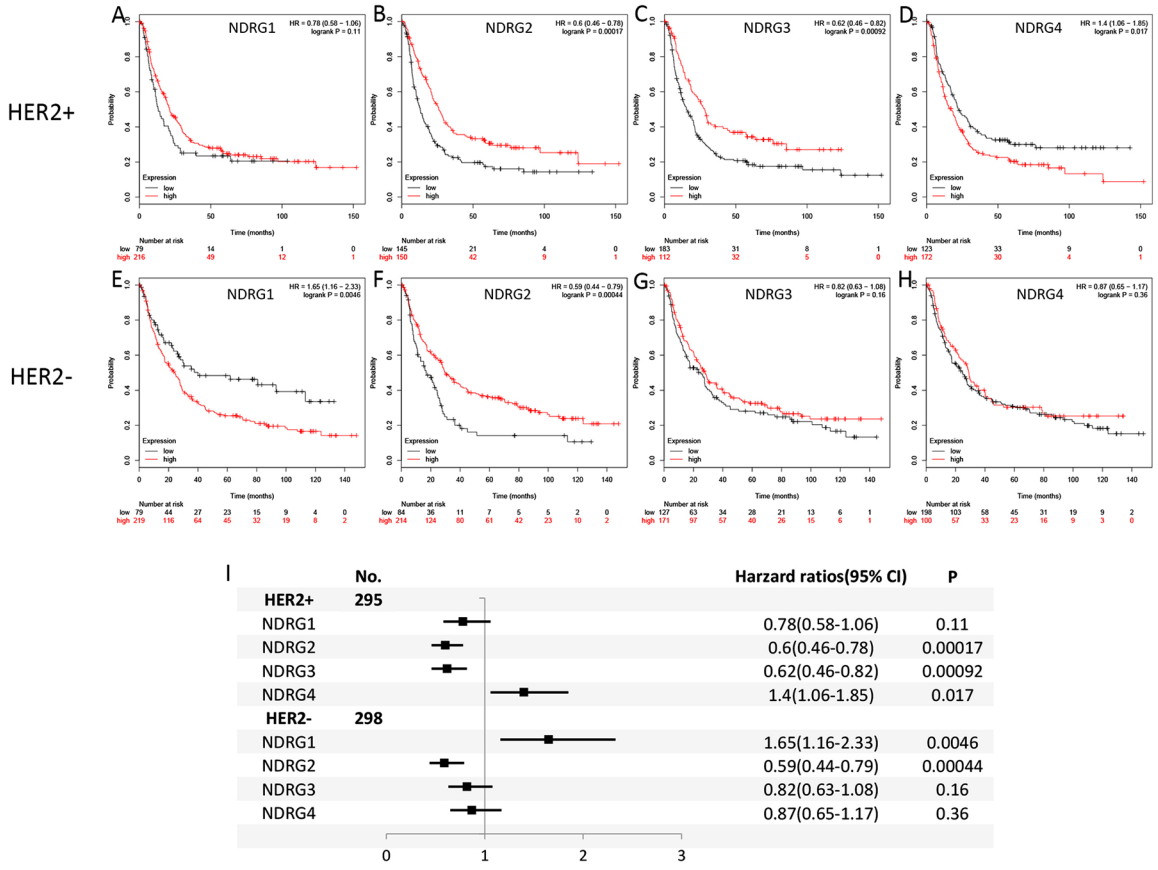

Figure 2. Survival curves of *NDRG* family members in *HER2*$^{+/-}$ subgroups. (a–h). Survival curves of *NDRG1* (Affymetrix IDs: 200632_s_at), *NDRG2* (Affymetrix IDs: 206453_s_at), *NDRG3* (Affymetrix IDs: 217286_s_at), *NDRG4* (Affymetrix IDs: 209159_s_at) are plotted for patients with *HER2*$^{+/-}$; (i) Forest plot of the prognostic HRs of *NDRG* family members in *HER2*$^{+/-}$ GC.
Red: high expression level; black: low expression level.
GC, gastric cancer; *HER2*, human epidermal growth factor receptor 2; HR: hazard ratio.

Prognostic value of NDRG *family signature*

For each individual in the STAD data of TCGA, a prognostic risk score was computed based on the risk score equation. Risk score = $\exp_{\text{mRNA of NDRG1}} \times -0.102 + \exp_{\text{mRNA of NDRG2}} \times 0.057 + \exp_{\text{mRNA of NDRG3}} \times 0.046 + \exp_{\text{mRNA of NDRG4}} \times 0.153$. All cases were assigned to the high/low-risk groups based on the score value with an optimal cutoff. In fact, distinct expression patterns of *NDRG* members were noticed between low ($n = 132$) and high-risk ($n = 220$) groups, particularly *NDRG1* and *NDRG4* [Figure 4(a, b)]. The low-risk group displayed a significantly favorable survival outcome than the high-risk group [Figure 4(c), HR = 1.76, 95% CI: 1.2–2.59, p value = 0.00385]. Of note, the ROC value increased to 0.679 as follow-up periods increased [Figure 4(d)].

The mRNA and protein expression of NDRG *family*

Next, we explored the mRNA and protein expression of the *NDRG* family between tumor and normal tissues. The mRNA expression of *NDRG2* was significantly reduced in tumor while the level of *NDRG3* was significantly elevated in tumor [Figure 5(a)]. Noteworthy, the entire *NDRG* family did not show diverse expression in stage-specific manner [Figure 5(b)]. The mRNA expression correlation among each *NDRG* member was comparably low, excluding potential direct correlational analysis [Figure 5(c)]. Moreover, the protein expression of *NDRG* members was also displayed [Figure 5(d)].

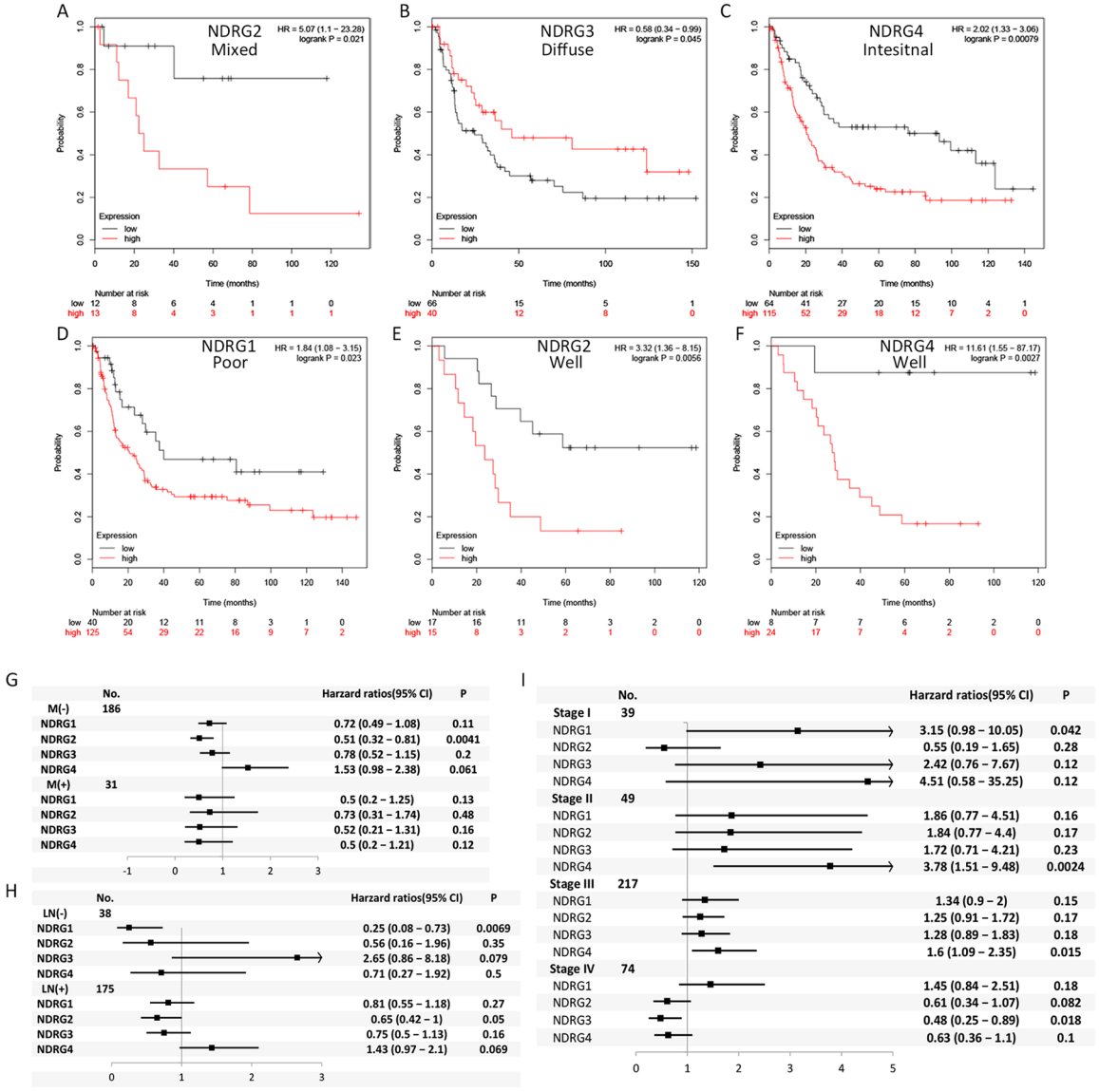

Figure 3. Survival curves of the *NDRG* family with different clinicopathological features. (a) Survival curve of *NDRG2* (Affymetrix IDs: 206453_s_at) with mixed type in Lauren classification; (b) survival curve of *NDRG3* (Affymetrix IDs: 217286_s_at) with diffuse type in the Lauren classification; (c) survival curve of *NDRG4* (Affymetrix IDs: 209159_s_at) with intestinal type in Lauren classification; (d) survival curve of *NDRG1* (Affymetrix IDs: 200632_s_at) with poor histological differentiation; (e) survival curve of *NDRG2* with well histological differentiation; (f) survival curve of *NDRG4* with well histological differentiation; (g) forest plots of the prognostic HR of *NDRG1–4* in GC with distant metastasis status; (h) forest plots of the prognostic HR of *NDRG1*-4 with lymph node status; (i) forest plots of the prognostic HR of *NDRG1–4* with pathological staging. Red: high expression level; black: low expression level.
GC, gastric cancer; HR, hazard ratio.

The mRNA and protein correlation between HER2*/EGFR and* NDRG1

Given the fact from Figure 2 that *NDRG1*, and *NDRG4* may feature inverse prognostic values in HER2$^{+/-}$ groups, we further explored the mRNA expression correlation between *NDRG1*, *NDGR4* and *HER2*. Moreover, given the close functional relationship between *HER2* and *EGFR*, *EGFR* was also included for correlational analysis. Interestingly, no significant result was identified [Figure 6(a–d)]. Next, the protein expression correlation was investigated in the RPPA data of

Table 1. The univariate and multivariate analysis of overall survival of *NDRG* family and clinical-pathological data from TCGA.

Characteristics	Univariate Cox			Multivariate Cox		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Sex	1.315	0.919–1.880	0.134	-	-	-
Age	1.615	1.105–2.360	0.013	1.967	1.333–2.903	0.001
T	1.769	1.154–2.713	0.009	1.364	0.839–2.218	0.211
N	2.076	1.365–3.157	0.001	1.53	0.88–2.660	0.132
Metastasis	2.194	1.261–3.817	0.005	1.851	1.033–3.315	0.038
Stage	2.025	1.420–2.889	<0.0001	1.388	0.830–2.322	0.211
NDRG1	1.015	0.853–1.206	0.868	–	–	–
NDRG2	1.09	0.932–1.276	0.281	–	–	–
NDRG3	0.895	0.645–1.243	0.51	–	–	–
NDRG4	1.12	0.998–1.258	0.055	–	–	–

CI, confidence interval; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.

Table 2. The univariate and multivariate analysis of recurrence-free survival of the *NDRG* family and clinical-pathological data from TCGA.

Characteristics	Univariate Cox			Multivariate Cox		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Sex	2.182	1.224–3.889	0.008	2.199	1.234–3.919	0.008
Age	0.869	0.527–1.433	0.582	–	–	–
T	0.87	0.512–1.480	0.608	–	–	–
N	1.246	0.730–2.129	0.42	–	–	–
Metastasis	1.259	0.457–3.471	0.656	–	–	–
Stage	1.042	0.640–1.696	0.87	–	–	–
NDRG1	1.245	0.956–1.621	0.104	–	–	–
NDRG2	1.067	0.851–1.337	0.575	–	–	–
NDRG3	0.824	0.499–1.361	0.449	–	–	–
NDRG4	1.241	1.053–1.462	0.01	1.247	1.057–1.470	0.009

CI, confidence interval; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.

TCGA. Of note, only *NDRG1*_pT346 was available. In fact, *NDRG1*_pT346 was significantly associated with EGFR ($r = -0.117$, $p = 0.02$), EGFR_pY1068 ($r = 0.218$, $p < 0.001$), EGFR_pY1173 ($r = -0.228$, $p < 0.001$), *HER2* ($r = 0.114$, $p = 0.024$) and *HER2*_pY1248

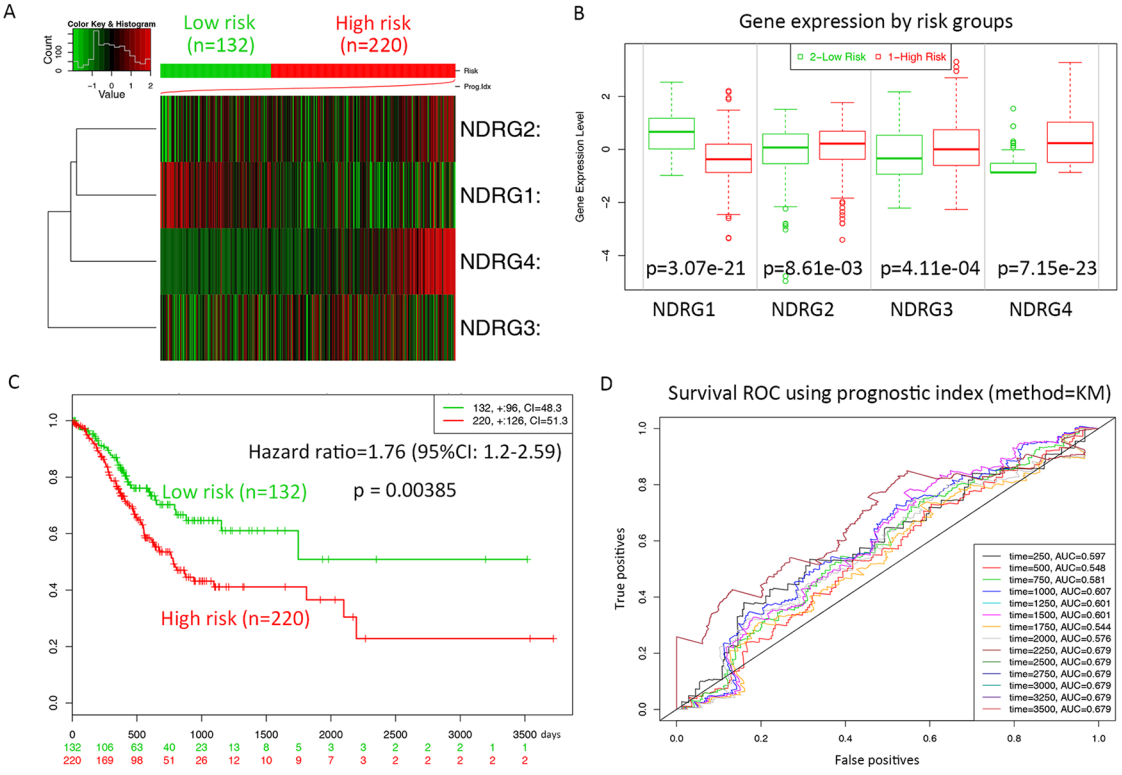

Figure 4. The prognostic values of the *NDRG* family signature. (a) Heat map for the clustered expression of the *NDRG* family between low (green, $n = 132$) and high (red, $n = 220$) risk groups; (b) Comparison of expression between low and high-risk groups for each *NDRG* member; (c) Survival curves of low and high-risk groups of the *NDRG* signature; (d) the ROC of survival curves over different times.
AUC, area under curve; CI, confidence interval; KM, Kaplan–Meier; Prog.Idx, prognostic index; ROC receiver operating characteristics.

[$r = 0.135$, $p = 0.008$; Figure 6(e–i)]. Moreover, the prognostic value of *NDRG1*_pT346 was analyzed. In fact, only high expression of *NDRG1*_pT346 showed a favorable OS ($p = 0.0014$).

GO enrichment analysis and genomic alterations of NDRG1-4

All the coexpressed genes of *NDRG1–4* (Pearson correlation $\geqslant 0.3$ or $\leqslant -0.3$) were annotated by GO and the KEGG pathway [Figure 7(a–d)]. In fact, epidermis development, extracellular exosome was the top significant biological processes (BP) and cellular components (CC) terms in *NDRG1* with no significant terms in molecular functions (MF) and KEGG [Figure 7(e)]. Mitotic nuclear division was the top BP of *NDRG2* with no significant results in CC, MF and KEGG [Figure 7(f)]. Mitotic cell process, nucleoplasm and adenyl nucleotide binding were the top significant BP, CC and MF terms in *NDRG3* with no significant term in KEGG [Figure 7(g)]. Epidermis development, cornified envelope and structural molecule activity were the top significant terms in BP, CC and MF in *NDRG4* with no significant term in KEGG [Figure 7(h)]. The genomic alterations of *NDRG1–4* included missense mutation, truncating mutation, amplification, deep deletion and mRNA upregulation [Figure 7(i)]. Moreover, given the relative weak mRNA correlation among each *NDRG* member, this study further explored potential TFs and miRNA that predicted to be connected with *NDRG* members. However, no miRNA or TF was predicted to synchronously correlate with all *NDRG* members or at least three of them [Figure 7(j, k)].

Prognostic values of NDRG1–4 *DNA methylation in MethSurv*

The DNA methylation levels of *NDRG1–4* with the prognostic values of each single CpG in TCGA were analyzed by MethSurv [Figure 8(a–d), Table 1]. In fact, cg15393676 of *NDRG1*, cg16409562 of *NDRG2*, cg26287101 of *NDRG3*

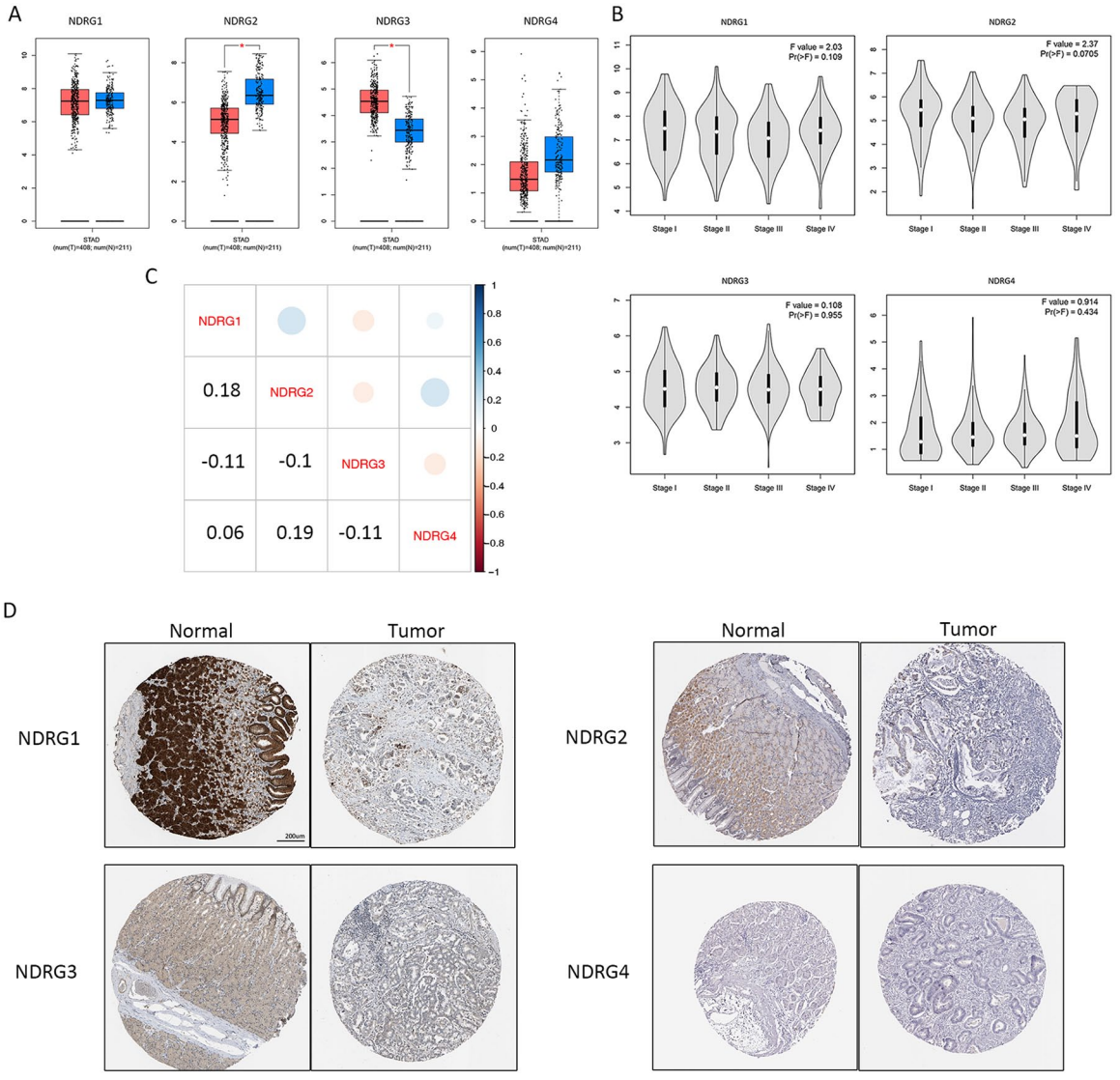

Figure 5. The mRNA and protein expression of *NDRG* family members. (a) The mRNA expression of *NDRG* family in tumor *versus* normal in STAD; red: tumor; blue: normal; (b) the mRNA expression of the *NDRG* family in different pathological stages; (c) the mRNA expression correlation among each *NDRG* member; (d) the protein expression of *NDRG* members in gastric cancer from the Human Protein Atlas.
STAD, stomach adenocarcinoma.

and cg00581595 of *NDRG4* showed the highest DNA methylation [Figure 8(A–D)]. Overall, 6 CpGs of *NDRG1*, 4 CpGs of *NDRG2*, 3 CpGs of *NDRG3* and 24 CpGs of *NDRG4* were associated with significant prognosis (Table 3).

Correlation between TIICs and NDRG *members*

Given the increasing association between immunological feature and prognosis in cancer, we further explored the correlation between TIICs and *NDRG* members. In fact, only $CD4^+$ T-cells showed the highest correlation with *NDRG4* [correlation = 0.341, $p = 2.14e^{-11}$; Figure 9(a)]. Given a variety of immune cells were defined by $CD4^+$ T-cells, we further examined the gene markers in each subtype [Figure 9(b–g)]. Of note *NDRG4* was highly associated with BCL6 in Tfh cells [correlation = 0.438, $p = 00e^{+}00$; Figure 9(c)].

Discussion

The increasing availability of published mRNA data, clinical outcomes and standardized analysis

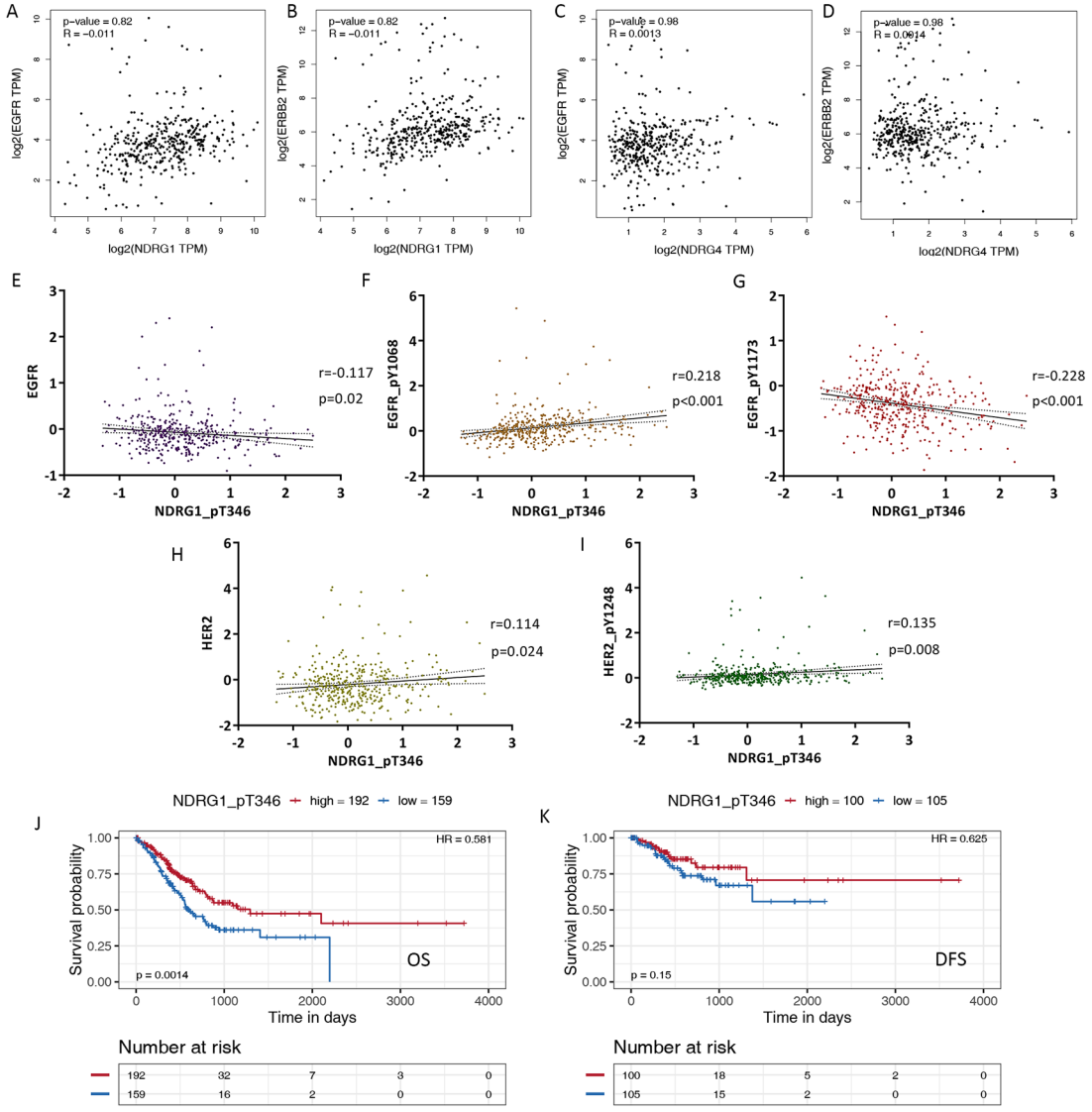

Figure 6. The mRNA and protein correlation between *HER2*/EGFR and *NDRG1* and the prognostic value assessment of *NDRG1*_pT346. (a–d) The mRNA correlation between *NDRG1*/*NDRG4* and *HER2*/EGFR in the STAD data of TCGA; (e–i) Correlations between *NDRG1*_pT346 and HER2, *HER2*_pY1248, EGFR, EGFR_pY1068 and EGFR_pY1173 in STAD of TCGA; (j) OS of prognostic value for *NDRG1*_pT346 in STAD of TCGA; (k) DFS of prognostic value for *NDRG1*_pT346 in STAD of TCGA.
Red: high expression; blue: low expression.
DFS, disease-free survival; EGFR, epidermal growth factor receptor; HR: hazard ratio; OS, overall survival; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.

platforms has provided the opportunities for exploring the correlation between gene expressions and type-specific cancer prognosis. This *in silico* study demonstrated distinct prognostic and biological values of *NDRG* family members in GC with mRNA expression and DNA methylation based on multiple cohorts from KM plotter and TCGA.

NDRG1 had been implicated in the regulation of embryonic placentation and organ development,[7] the cellular vesicle transport system,[13] endocytosis and recycling of membrane proteins.[14,15] The reduced expression of *NDRG1* had been associated with a worse prognosis in esophageal,[20] colorectal[21] and breast cancers[12] while leading to favorable clinical outcomes in hepatocellular carcinoma.[22] This paradoxical fact may be tumor type-specific, further highlighting the complicated biological function and processes that *NDRG1* is involved with. In fact, *NDRG1* was associated with

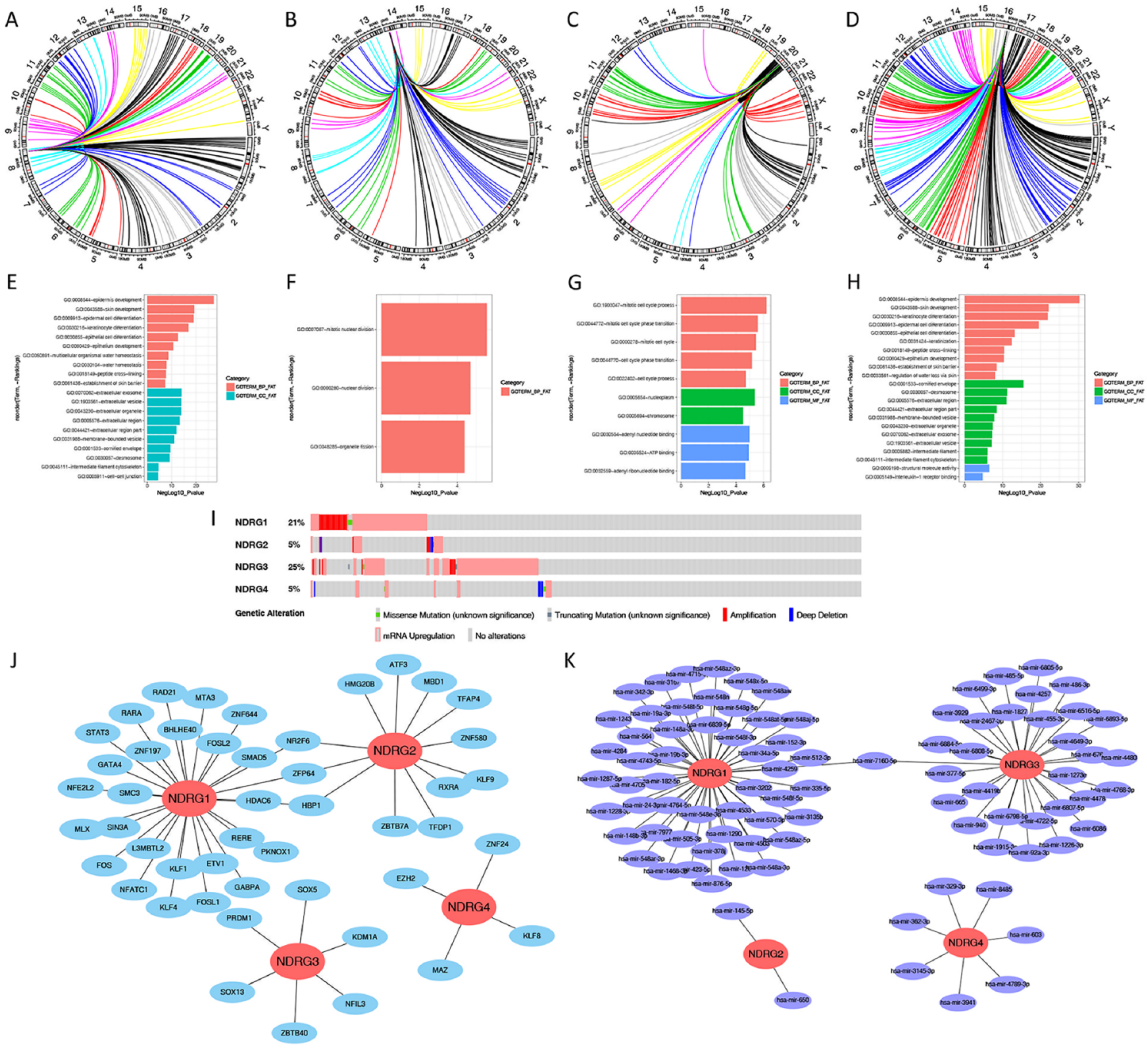

Figure 7. GO enrichment, genomic alterations and miRNA/TFs prediction of *NDRG1–4*. (a–d) Coexpressed genes associated with *NDRG1–4* (Pearson correlation ⩾0.3 or ⩽−0.3); the chromosomal positions of all genes coexpressed with *NDRG1–4* were displayed using various colorful lines; (e–h) GO enrichment for coexpressed genes associated with *NDRG1–4*; (e) GO enrichment for coexpressed genes with *NDRG1*; red: BP terms; green: CC terms; (f) GO enrichment for coexpressed genes with *NDRG2*; red: BP terms; (g) GO enrichment for coexpressed genes with *NDRG3*; red: BP terms; green: CC terms; blue: MF; (h) GO enrichment for coexpressed genes with *NDRG4*; BP terms; green: CC terms; blue: MF terms; (i) the genomic alterations of *NDRG1–4* in STAD of TCGA; green in grey: missense mutation (unknown significance); darker grey in grey: truncating mutation (unknown significance); red: amplification; blue: deep deletion; pink circle in grey: mRNA upregulation; grey only: no alteration; (j) The predicted networks of TFs and *NDRG1–4*; red: *NDRG* family; light blue: predicted TFs; line: predicted interactions; (k) the predicted networks of miRNAs and *NDRG1–4*, red, *NDRG* family; violet, predicted miRNA; line, predicted interactions.

BP, biological process; CC, cellular components; GO, gene ontology; MF, molecular functions; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TF, transcription factor.

a decrease in the proliferation and induction of apoptosis of cancer cells by the regulation of Bcl-2 and Ca^{2+}-associated protein 43,[48,49] and the dysregulation of epithelial-mesenchymal transition (EMT).[50–52] Nonetheless, *NDRG1* might exert inconsistent effects in GC prognosis.[49,50,53,54]

In this study, although *NDRG1* was not significantly associated with overall prognosis in all cases, 6 CpGs of *NDRG1* were associated with significant prognosis. Of note, inverse prognostic values of *NDRG1* in $HER2^{+/-}$ groups indicated a potential correlation between *NDRG1* and *HER2*.

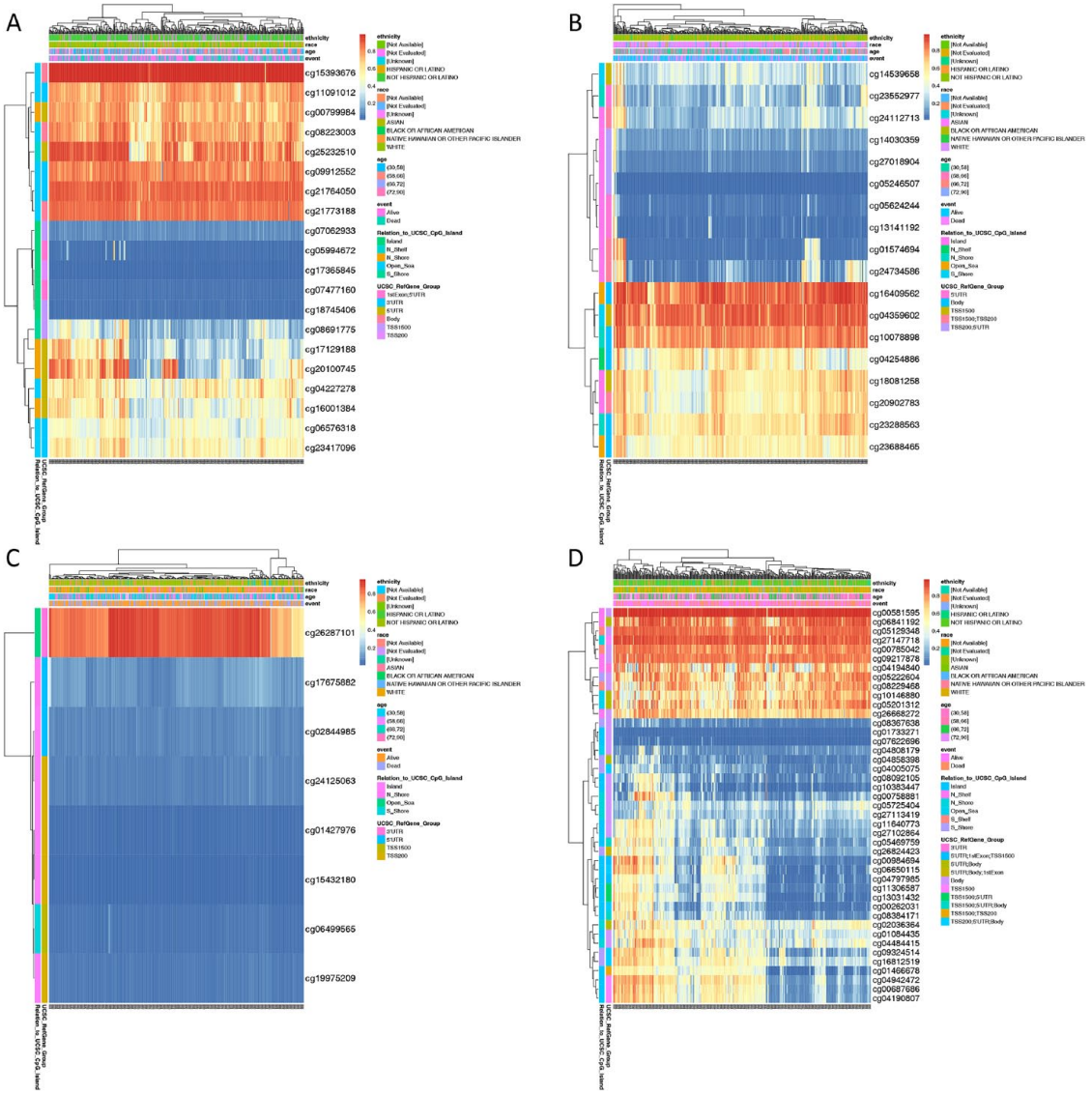

Figure 8. DNA methylation of *NDRG1–4* in MethSurv. (a–d) The DNA methylation levels of *NDRG1–4*; (a) the DNA methylation clustered expression of *NDRG1*; (b) The DNA methylation clustered expression of *NDRG2*; (c) The DNA methylation clustered expression of *NDRG3*; (d) The DNA methylation clustered expression of *NDRG4*; Red to blue: high expression to low expression. Various colorful side boxes were used to characterize the ethnicity, race, age, event, relation to UCSC_CpG_island, UCSC_refGene_Group.

However, no significant mRNA expression correlation was identified between *NDRG1* and *HER2*/EGFR. Furthermore, the protein expression of *NDRG1* phosphorylation level, *NDRG1*_pT346, was found to be significantly associated with EGFR, EGFR_pY1068, EGFR_pY1173, *HER2* and *HER2*_pY1248. Previous study indicated that, in colon cancer and pancreatic cancer, *NDRG1* significantly reduced the expression of *HER2* (general expression, heterodimerization and phosphorylation) and the activation of downstream MAPKK in response to the epidermal growth factor ligand.[55] For the first time, our study highlighted a potential role of *NDRG1* associated with *HER2* status in GC. Given the *HER2* targeting drug, trastuzumab, has been widely used in GC,[56] digging into the *NDRG1*-related mechanisms may shed light upon further biological and pharmacological values.

Collectively, although *NDRG1* was not validated as an independent general prognostic factor in

Table 3. The significantly prognostic values of CpG in the *NDRG* family.
The prognostic values of CpG in the *NDRG* family by MethSurv ($p < 0.05$).

Gene-CpG	HR	LR test *p* value
NDRG1 – 1stExon;5′UTR–Island–cg05994672	1.392	0.048
NDRG1 – 3′UTR–Open_Sea–cg09912552	0.629	0.0052
NDRG1 – 3′UTR–Open_Sea–cg21764050	0.687	0.042
NDRG1 – 5′UTR–N_Shore–cg16001384	0.68	0.028
NDRG1 – TSS1500–Island–cg07062933	1.622	0.014
NDRG1 – TSS200–Island–cg17365845	1.433	0.038
NDRG2 – Body–N_Shelf–cg04254886	1.715	0.0051
NDRG2 – TSS1500–S_Shore–cg04359602	0.719	0.045
NDRG2 – TSS200;5′UTR–Island–cg05246507	0.672	0.02
NDRG2 – 5′UTR.Island.cg13141192	0.647	0.014
NDRG3 – 3′UTR–Open_Sea–cg26287101	0.675	0.029
NDRG3 – 5′UTR–Island–cg02844985	1.519	0.033
NDRG3 – 5′UTR–N_Shore–cg17675882	1.49	0.029
NDRG4 – 5′UTR;1stExon; TSS1500–Island–cg00984694	0.618	0.019
NDRG4 – 5′UTR;1stExon; TSS1500–Island–cg04797985	0.603	0.015
NDRG4 – 5′UTR; Body;1stExon–S_Shore–cg26824423	0.67	0.026
NDRG4 – 5′UTR; Body–S_Shore–cg04858398	0.653	0.034
NDRG4 – Body–Island–cg01084435	0.595	0.0023
NDRG4 – Body–Island–cg08092105	0.644	0.026
NDRG4 – Body–Island–cg10383447	0.596	0.0061
NDRG4 – Body–Island–cg11640773	0.594	0.0024
NDRG4 – Body–Island–cg27102864	0.648	0.0098
NDRG4 – Body–N_Shore–cg04484415	0.642	0.017
NDRG4 – Body–S_Shelf–cg00785042	0.591	0.0084
NDRG4 – Body–S_Shelf–cg05129348	0.585	0.0019
NDRG4 – TSS1500;5′UTR; Body–Island–cg05469759	0.637	0.0079
NDRG4 – TSS1500;5′UTR; Body–Island–cg08384171	0.659	0.02
NDRG4 – TSS1500; 5′UTR–Island–cg13031432	0.632	0.024
NDRG4 – TSS1500–Island–cg00687686	0.698	0.029
NDRG4 – TSS1500–Island–cg04190807	0.647	0.0084

Table 3. (Continued)

Gene-CpG	HR	LR test *p* value
NDRG4 – TSS1500–Island–cg04942472	0.641	0.0066
NDRG4 – TSS1500–N_Shore–cg27147718	0.558	0.0047
NDRG4 – TSS200;5′UTR; Body–Island–cg00262031	0.582	0.0012
NDRG4 – TSS200;5′UTR; Body–Island–cg06650115	0.689	0.025
NDRG4 – TSS200;5′UTR; Body–S_Shore–cg04005075	0.676	0.018
NDRG4 – TSS200;5′UTR; Body–S_Shore–cg09324514	0.538	0.00065
NDRG4 – TSS200;5′UTR; Body–S_Shore–cg16812519	0.662	0.013

HR, hazard ratio; LR, log-rank.

multivariate analysis from TCGA, the prognostic value of *NDRG1* was highlighted in GC subsets with significant correlation to *HER2*.

The *NDRG2* expression had been found significantly reduced in pancreatic, breast cancers and hepatocellular carcinoma compared with normal tissues, accompanied by more aggressive features and a high ratio of relapse.[23–25] In this study, *NDRG2* was associated with favorable prognosis in all and significantly reduced in tumors compared with normal tissues, consistent with previous studies.[26] However, no significance was found in the stage-specific pattern. Of note, increased chemo-resistance and decreased Fas-mediated cell death had been validated due to the inhibition of *NDRG2*.[26] Interestingly, the promoter methylation of *NDRG2* was frequently hypermethylated, leading to decreasing expression of *NDRG2* at both mRNA and protein level and further associated with worse prognosis of GC.[57] Similar prognostic role of *NDRG2* had been validated in prostate cancer. The downregulation of *NDRG2* was associated with advanced pathological stages and identified as an independent prognostic factor for short recurrence-free survival and OS.[27] Of note, overexpression of *NDRG2* could decrease the radiosensitization of Hela cells by the regulation of Bax signaling.[58]

NDRG3 was found to be significantly upregulated in prostate cancer, and was associated with advanced pathological stage and a worse prognosis of prostate cancer, contrary to *NDRG2*.[27] Currently the role of *NDRG3* had not been fully investigated in GC. Our study had revealed that *NDRG3* was significantly associated with a favorable prognosis for the OS of all patients, as well as the *HER2*$^{+}$ and diffuse type subgroups. Furthermore, *NDRG3* was significantly increased in tumor compared with normal, with no significant distribution in various stages. In fact, although *NDRG3* showed a favorable outcome based on the outcome from the KM plotter, a significant upregulation of *NDRG3* was found in tumors compared with normal tissues in TCGA. Moreover, upregulation of *NDRG3* was also associated with the high-risk group in the *NDRG* signature. There are a few issues that need to be clarified. First, clinical heterogeneity may account for the controversial outcome between TCGA and KM plotter (GSE14210, GSE15459, GSE22377, GSE29272, GSE51105 and GSE62254). Second, multivariate Cox analysis of the *NDRG* family using TCGA also eluded the potential independent prognostic value of *NDRG3*, both in OS and RFS. However, given the current studies remained sparse, the biological and prognostic values of *NDRG3* warrant further intensive investigation. It may be insightful to systematically explore the prognostic value of *NDRG3* using meta-analysis.

Previously, *NDRG4* was found reduced in both mRNA and protein expression in colorectal cancer tissues compared with normal counterparts, and significantly suppressed tumor invasion and proliferation.[28] However, it was not significantly reduced in tumors compared with normal tissues in GC from this study. Similar to *NDRG3*, the role of *NDRG4* had not been clear. Moreover, 24 CpGs of *NDRG4* exhibited significant prognostic values.

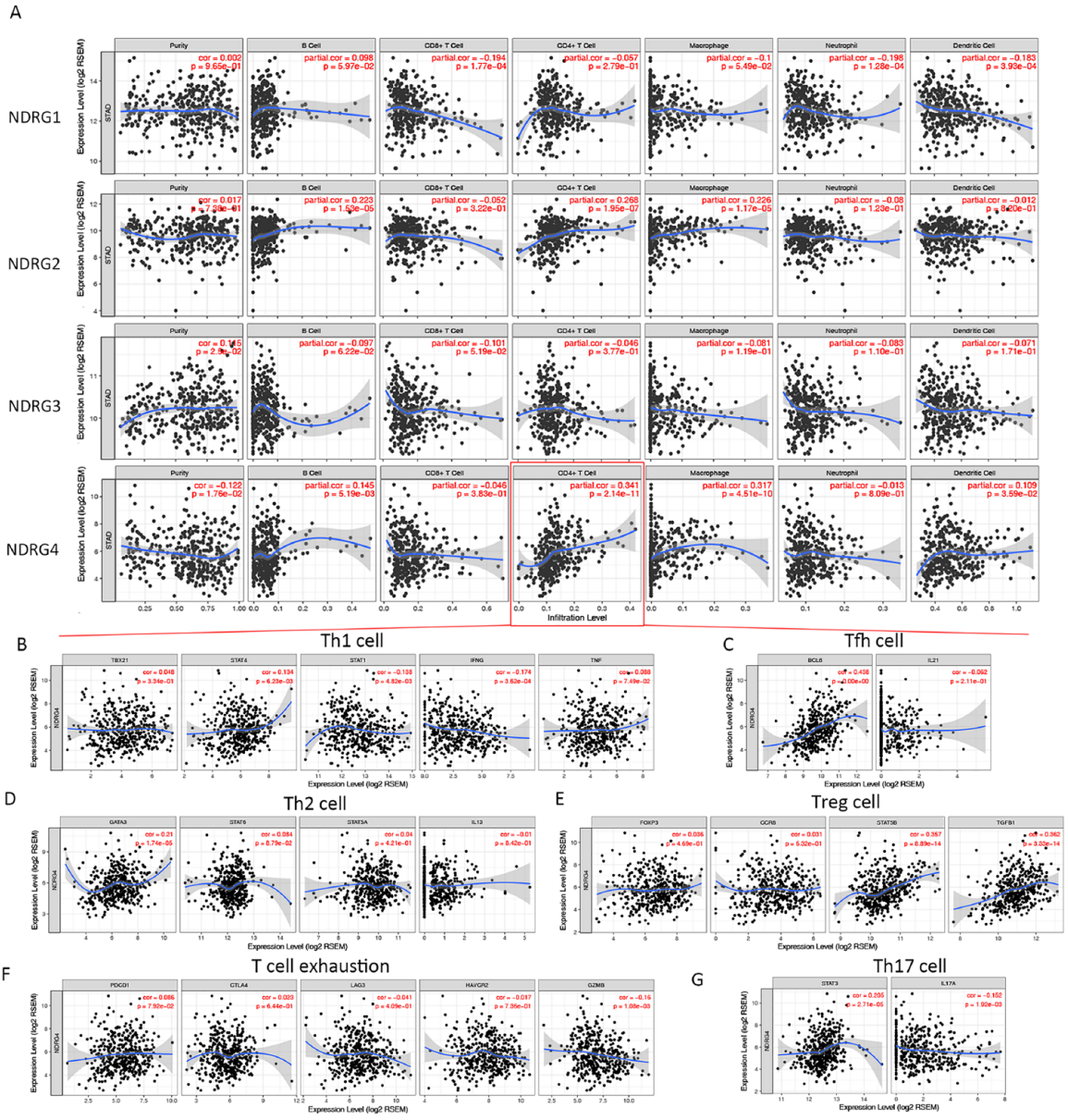

Figure 9. Correlation analysis between *NDRG* members and TIICs. (a) The correlation between each type of TIICs (B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, neutrophils, macrophages and dendritic cells) and *NDRG* family; (b) The correlation between the gene markers [*TBX21* (*T-bet*), *STAT4, STAT1, IFNG* (interferon gamma) and *TNF* (tumor necrosis factor alpha)] of Th1 and *NDRG4*; (c) the correlation between the genes markers (*BCL6, IL21*) of Tfh cells and *NDRG4*; (d) the correlation between the genes markers (*GATA3, STAT6, STAT5A* and *IL13*) of Th2 cells and *NDRG4*; (e) the correlation between the genes markers [*FOXP3, CCR8, STAT5B* and *TGFB1* (transforming growth factor beta)] of Treg cells and *NDRG4*; (f) the correlation between gene markers [*PDCD1* (programmed cell death 1), *CTLA4, LAG3, HAVCR2 (TIM-3)* and *GZMB*)] of T-cell exhaustion and *NDRG4*; (g) the correlation between gene markers (*STAT3* and *IL17A*) of Th17 cells and *NDRG4*.
IL, interleukin; TIICs, tumor infiltrating immune cells; STAT4, signal transducer and activator of transcription 4; BCL6, B-Cell Lymphoma 6; IL21, interleukin 21; GATA3, GATA binding protein 3; FOXP3, forkhead box P3; CCR8, C-C Motif Chemokine Receptor 8; TGFB1, Transforming Growth Factor Beta 1; CTLA4, cluster of differentiation 152; LAG3, Lymphocyte activation gene 3; HAVCR2. TIM-3, Hepatitis A Virus Cellular Receptor 2; GZMB, Granzyme B..

Despite the correlation between *NDRG4* and *HER2*, mRNA expression was not significant based on TCGA data, it was perceived that *NDRG4* could exert effects on the downstream signaling components of *HER2*, such as RAS/RAF/MAPK/ERK. Interestingly, only *NDRG4* was validated as

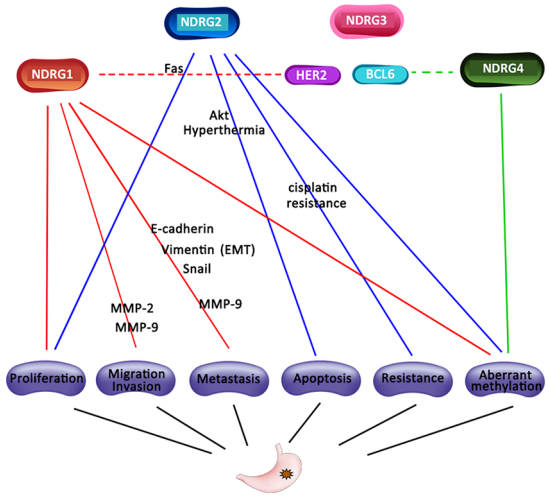

Figure 10. Regulation network of *NDRG* family in GC. Red: *NDRG1*-associated mechanism; blue: *NDRG2*-associated mechanism; pink: *NDRG3*-associated mechanism; green: *NDRG4*-associated mechanism; dash line indicated predicted correlation. EMT, epithelial-mesenchymal transition; GC, gastric cancer.

an independent prognostic factor in TCGA dataset, further indicating a possible association between *NDRG4* and recurrence in GC. For the rest of the *NDRG* family, it remained far from conclusive due to possible race diversity and other confounding factors such as radiochemotherapy.

Interestingly, although *NDRG1* and *NDRG4* did not show significantly differential expression between tumor and normal tissues in STAD using the GEPIA platform, the high/low-risk groups exhibited distinct expression patterns of *NDRG1* and *NDRG4* using the same dataset [Figure 4(a, b)]. In fact, the *NDRG* member signature may provide insightful clues on the prognostic values of combinational analysis, rather than a single gene.

The current network regulation of the *NDRG* family associated with GC was summarized (Figure 10).[26,50,53–55,57,59–64] *NDRG1, 2* and *4* have been reported to feature aberrant methylation in GC compared with normal tissues.[57,63,64] Reduced expression of *NDRG1* was associated with enhanced migration, invasion and metastasis *via* several mechanisms, including *EMT, MMP-2* and *MMP-9*.[50,53,54,59,60] Although *NDRG2* was not an independent prognostic indicator in this manuscript, it was determined as an independent risk factor by Choi and colleagues.[26] Moreover, silencing of *NDRG2* increased the proliferation and resistance of cisplatin in GC cell lines.[26] Up to now, studies focusing on the association between *NDRG3* and *4* and GC remain limited. Interestingly, in this manuscript, *NDRG4* was highly associated with *BCL6* in Tfh cells. Up to now, this is the first study reporting the correlation between *BCL6* and *NDRG4* in GC, indicating a potential role of *NDRG4* in follicular helper $CD4^+$ T-cells. Moreover, potential inverse prognostic values of *NDRG1* between *HER2*$^{+/-}$ GC and the significant association between protein expression of *NDRG1* (*NDRG1*_pT346) and *HER2*/*HER2*_pY1248 indicated possible connection as well. However, *in silico* findings warrant further experimental validation.

Up to now, methylation-related study of the *NDRG* family remains limited. High levels of *NDRG1* promoter methylation in the CpG islands were found in both GC cell lines and tissues.[64] Interestingly, no mutation of *NDRG1* was detected in this study.[64] Consistently, our finding also indicated rare cases of *NDRG1* mutation. For *NDRG2*, hypermethylation status was detected in the *NDRG2* promoter both in GC cell lines and tissues.[62] In fact, the reduced expression of *NDRG2* in GC compared with normal tissues was highly correlated with the promoter hypermethylation.[62] For *NDRG4*, both promoter and gene body methylation levels were increased in GC tissues.[63] Interestingly, opposite clinical results of *NDRG4* were found between the Chinese samples from Chen and colleagues and TCGA data, highlighting the race difference beneath the prognostic values of *NDRG4*.[63]

The limitation of this study was the lack of experimental validation and externally clinical cohort validation. The limited number of some subgroups of KM plotter for prognostic analysis and potential sample heterogeneity could bias the results. Further validation on a larger sample size is also required.

Conclusions

This *in silico* study investigated the biological and prognostic values of the *NDRG* family in GC based on KM plotter and TCGA, providing insights for further investigation of *NDRG* family as potential targets in GC.

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The contributions of authors are as follows:
CY, XH, SZ, WH and JS carried out data analysis.
CY, XH, JL and MZ drafted the manuscript.
MZ, JS and CY participated in study design, data collection and analysis.
MZ, JS and CY revised the manuscript.
All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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