Biochemical and Biophysical Research Communications 439 (2013) 241–246

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Correlation of N-myc downstream-regulated gene 1 subcellular localization and lymph node metastases of colorectal neoplasms

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ARTICLE INFO

Article history:
Received 13 August 2013
Available online 22 August 2013

Keywords:
Colorectal cancer
N-myc downstream-regulated gene 1
Subcellular localization
Lymph node metastasis

ABSTRACT

In colorectal neoplasms, N-myc downstream-regulated gene 1 (NDRG1) is a primarily cytoplasmic protein, but it is also expressed on the cell membrane and in the nucleus. NDRG1 is involved in various stages of tumor development in colorectal cancer, and it is possible that the different subcellular localizations may determine the function of NDRG1 protein. Here, we attempt to clarify the characteristics of NDRG1 protein subcellular localization during the progression of colorectal cancer. We examined NDRG1 expression in 49 colorectal cancer patients in cancerous, non-cancerous, and corresponding lymph node tissues. Cytoplasmic and membrane NDRG1 expression was higher in the lymph nodes with metastases than in those without metastases ($P < 0.01$). Nuclear NDRG1 expression in colorectal neoplasms was significantly higher than in the normal colorectal mucosa, and yet the normal colorectal mucosa showed no nuclear expression. Furthermore, our results showed higher cytoplasmic NDRG1 expression was better for differentiation, and higher membrane NDRG1 expression resulted in a greater possibility of lymph node metastasis. These data indicate that a certain relationship between the cytoplasmic and membrane expression of NDRG1 in lymph nodes exists with lymph node metastasis. NDRG1 expression may translocate from the membrane of the colorectal cancer cells to the nucleus, where it is involved in lymph node metastasis. Combination analysis of NDRG1 subcellular expression and clinical variables will help predict the incidence of lymph node metastasis.

1. Introduction

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, and over 1.2 million new cancer cases and 608,700 deaths occurred from colorectal cancer in 2008 [1]. Despite advances in surgical and nonsurgical therapies, colorectal tumors are still the third major cause of cancer-related mortality [2]. Traditional therapies have focused on treating the symptoms and not the cause of the disease. Thus, new treatments for colorectal cancer have focused on the important molecules involved in disease progression.

N-myc downstream-regulated gene 1 (NDRG1), also known as Drg1, Cap43, RTP, Rit42, and PROXY-1, is a member of the NDRG gene family. NDRG1 is a highly conserved protein between mouse and human, and it is comprised of a 394 amino acid polypeptide, with a molecular weight of 43 kDa [3,4]. NDRG1 is a multifunctional protein involved in tumorigenesis and tumor development, and its function differs in different tumor types. In colorectal, prostate, cervical, and ovarian cancers, NDRG1 plays important roles in metastasis suppression [5–8], while in hepatocellular carcinomas, NDRG1 enhances portal vein invasion and intrahepatic metastasis [9,10]. NDRG1 expression has been correlated with various clinicopathological features, such as histopathological type, tumor differentiation, Dukes' stage, lymphatic invasion, and venous invasion, but these correlations may depend on the race or ethnicity of the patients [11–13]. NDRG1 has long been defined as a metastasis suppressor gene in colorectal cancer. Numerous clinical and scientific studies have explored the possible mechanisms by which NDRG1 inhibits metastasis.

Recently, more and more studies have focused on the correlation between the subcellular localization of molecules and tumor development. Generally, these molecules perform different functions depending on their subcellular localization. SIRT1, a well-studied protein that has different subcellular localizations,

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0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.08.049

Table 1
Characteristics of the study population.

Categorical variables	*n* = 49 (%)
Sex	
Female	20 (40.8)
Male	29 (59.1)
Age	
Mean ± SD	54.3 ± 13.1
Tumor distribution	
Rectum	25 (51.0)
Colon	24 (49.0)
Pathological grading	
II	16 (32.7)
II–III	10 (20.4)
III	23 (46.9)
Lymph node metastasis	
Yes (N1 + N2)	37 (75.5)
No (N0)	12 (24.5)

was reviewed by Song and Surh [14]. SIRT1 has both positive and negative effects on carcinogenesis partially due to its subcellular localization. The nuclear or cytoplasmic localization of SIRT1 affected its substrate specificity in both normal and cancer cells. Furjelova et al. clarified the significant differences of CA IX expression that depend on subcellular localization in fibroadenomas and carcinomas; they confirmed that CA IX was a valuable biomarker for diagnosis and prognosis determination in breast cancer [15]. Sun et al. reported that nuclear and cytoplasmic HuR expression were correlated with different adverse phenotypes in Gallbladder carcinoma, and cytoplasmic HuR expression is an independent prognostic factor for poor disease-free survival [16]. In colorectal cancer, NDRG1 protein is expressed in the cytoplasm, on the membrane, and in the nucleus [17–19]. However, clinical data clarifying the changes of NDRG1 subcellular distribution during the progression of colorectal cancer are rare. Different subcellular localization in normal cells, cancer cells, and metastatic cells may determine the function of NDRG1 protein. Here, we explored NDRG1 subcellular expression in normal colorectal mucosa, primary colorectal neoplasms, and metastatic sites in the regional lymph nodes.

2. Materials and methods

2.1. Tissue samples

The tissue microarray was purchased from Shanghai Outdo Biotech Co., LTD, Shanghai, China and included 49 colorectal cancer specimens of cancerous, non-cancerous, and corresponding lymph node tissues. Among them, 37 cases were diagnosed as positive lymph node metastases, and 12 cases were negative. Table 1 describes the patient characteristics of the study population.

2.2. Immunohistochemistry

Immunohistochemical (IHC) staining was performed directly on the tissue microarray. NDRG1 goat polyclonal antibody (Pierce, Thermo Fisher Scientific, USA) was used at a 1:100 final dilution. Briefly, the slides were treated with 3% H_2O_2 for 15 min to block endogenous peroxidase activity. Then, the slides were incubated with 10% normal goat serum to block nonspecific binding sites. Thereafter, the slides were incubated with the primary antibody overnight at 4° C. After washing, peroxidase-conjugated Affinipure rabbit anti-goat IgG (ZSGB-Bio, China) was applied at a 1:1000 dilution for 10 min at 37° C. S-P complex was added at room temperature for 30 min, and diaminobenzidine was used as the final chromogen. Finally, the slides were counterstained with hematoxylin. All of the immunohistochemical images were captured using an Aperio pathological scanning system.

Immunoreactivity for NDRG1 expression was independently assessed by two well-known pathologists from Shanghai Outdo Biotech Company. Patients who died or did not follow up were excluded from this study. In total, 41 cases were adopted in our study. The cell staining reaction was estimated according to the revolutionary immunoreactive score (IRS) method: IRS = SI (staining intensity) × PP (percentage of positive cells). SI was determined as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). PP was defined as the exact percentage of positive cells.

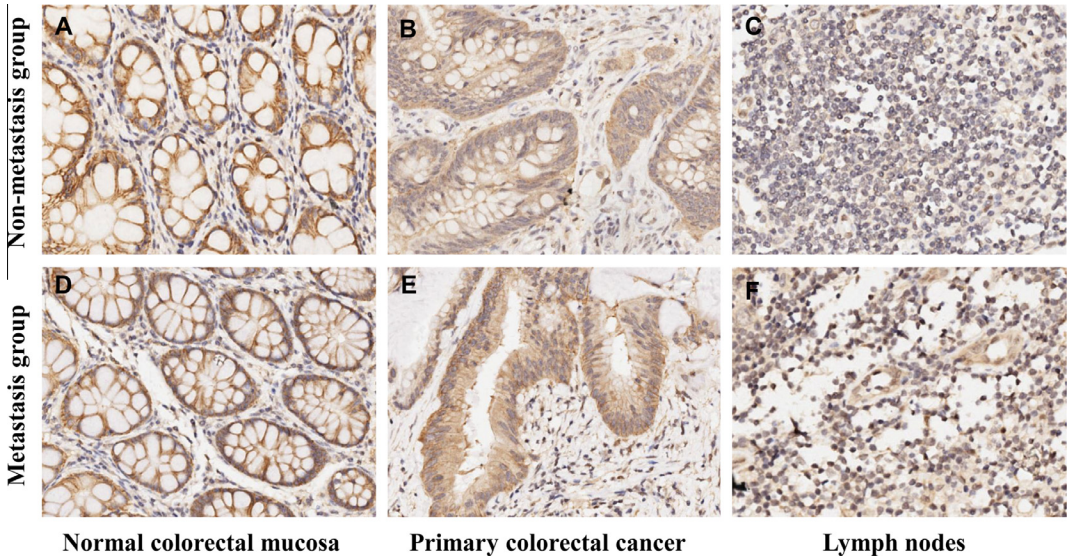

Fig. 1. Immunohistochemical staining of NDRG1 in human colorectal tissues. (A–C), representative images of lymph node samples without metastases. (D–F), representative images of lymph node samples with metastases. (A and D) Expression of NDRG1 in normal colorectal mucosa. (B and E) Expression of NDRG1 in primary colorectal cancers. (C and F) Expression of NDRG1 in the corresponding regional lymph nodes.

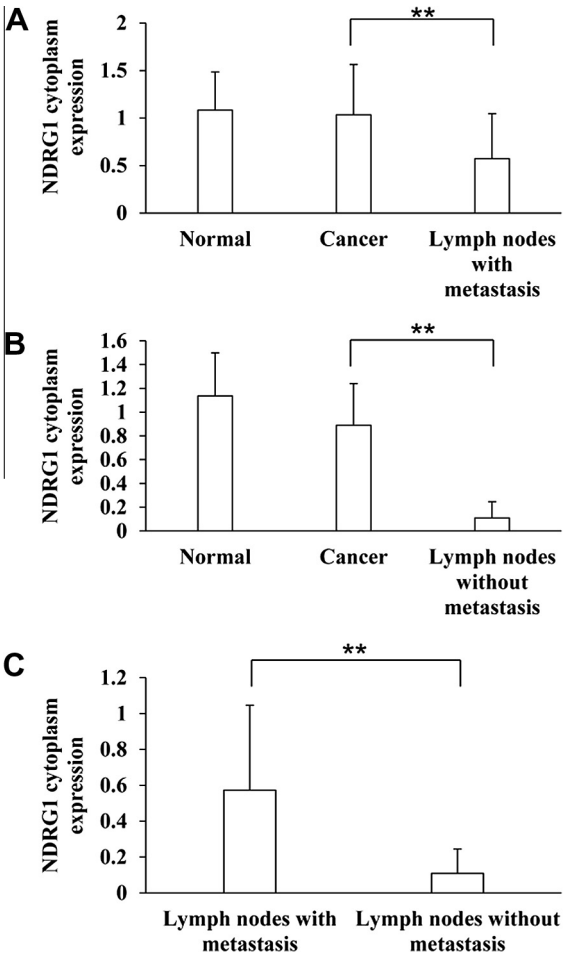

Fig. 2. Cytoplasmic NDRG1 expression in the different groups. (A) NDRG1 expression was significantly higher in the colorectal neoplasms than in the lymph nodes with metastases ($P < 0.01$). (B) NDRG1 expression was significantly higher in the colorectal neoplasms than in the lymph nodes without metastases ($P < 0.01$). (C) NDRG1 expression in the lymph nodes with metastases was higher than in the lymph nodes without metastases ($P < 0.01$). *P value <0.05, **P value <0.01.

2.3. Statistical analysis

The SPSS 19.0 software was used for the statistical analyses. Differences in NDRG1 expression between the different groups were compared using a rank sum test. Spearman's correlation coefficient analysis was used to evaluate the relationship between NDRG1 expression and the clinical variables. All reported P values were two-sided, and $P < 0.01$ or $P < 0.05$ was considered statistically significant.

3. Results

3.1. NDRG1 expression in the cytoplasm of colorectal cancer cells

Immunoreactivity was primarily observed in the cytoplasm. Colorectal cancer, adjacent normal colorectal mucosa, and the corresponding regional lymph nodes were all positive for cytoplasmic NDRG1 (Fig. 1). NDRG1 expression was significantly higher in the primary colorectal neoplasms than in the regional lymph nodes with ($P < 0.01$; Fig. 2A) or without metastases ($P < 0.01$; Fig. 2B), and the expression of NDRG1 in the lymph nodes with metastases was higher than in those without metastases ($P < 0.01$; Fig. 2C). Furthermore, in the patients with lymph nodes metastases, there was a negative correlation between lymph node staining and histopathological grading ($P < 0.01$; $r_s = -0.401$; Fig. 3A). In other words, higher levels of NDRG1 protein expression in the positive lymph nodes correlated with the differentiation of the colorectal neoplasm.

3.2. NDRG1 expression in the membrane of colorectal cancer cells

There was a significantly lower expression in the membrane of the colorectal neoplasms than in the normal colorectal mucosa ($P < 0.01$; Fig. 4A and B) and in the patients without lymph nodes metastases. NDRG1 membrane expression in the colorectal neoplasms was higher than in the metastatic lymph nodes ($P < 0.01$; Fig. 4B). As in the cytoplasm, NDRG1 membrane expression was also higher in the lymph nodes with metastases than in those without metastases ($P < 0.01$; Fig. 4C). It is worth mentioning that there was no positive staining in the membrane of lymph nodes without metastasis. In the group with lymph node metastases, there was a good correlation between cytoplasmic NDRG1 expression in the colorectal neoplasms and the membrane expression in the lymph nodes ($P < 0.05$; $r_s = 0.402$; Fig. 3B), and NDRG1 membrane expression in the lymph nodes was positively correlated with TNM classification ($P < 0.05$; $r_s = 0.446$; Fig. 3C) and the proportion of positive lymph nodes ($P < 0.05$; $r_s = 0.395$; Fig. 3D).

3.3. NDRG1 expression in the nucleus of colorectal cancer cells

There was no observed staining in the nucleus of normal colorectal mucosa or colorectal neoplasms. In the corresponding regional lymph nodes, the immunoreactivity was relatively weaker than that of the cytoplasm and the membrane. In the group with lymph node metastases, nuclear NDRG1 expression in the colorectal neoplasms was significantly higher than in the normal colorectal mucosa ($P < 0.01$; Fig. 4D).

4. Discussion

NDRG1 is a predominantly cytoplasmic protein, but it is also expressed in the cell membrane and nucleus. In the cytoplasm, NDRG1 protein immunoreactivity was localized to the inner membrane of the mitochondria, even though it lacks a mitochondrial targeting sequence. However, the single phosphopantetheine attachment site motif may help NDRG1 protein localize in the mitochondria [19]. NDRG1 was also found to be adjacent to the adherens junctions and desmosomes within the cytoplasm. There is a highly hydrophobic 20 amino acid sequence in the NDRG1 protein sequence that may explain its behavior as a membrane-associated protein [20]. Other studies found that NDRG1 expression was localized in the nucleolus [19]. However, there were no nuclear targeting sequences identified in the NDRG1 protein sequence.

Cytoplasmic NDRG1 expression was higher in the primary colorectal neoplasms than in the regional lymph nodes with metastases in this study; these data were in accordance with an *in vitro* study that reported that NDRG1 expression was much higher in SW480 cells (established from a primary colorectal tumor) than in SW620 cells (established from a lymph node metastasis from the same patient a year later) [5]. The results of that study indicated that NDRG1 downregulation in SW620 cells was associated with a decrease in unique histone modifications. This was also the case in the patients without lymph nodes metastases. Thus, it is possible that NDRG1 downregulation in the corresponding lymph node tissues was not directly correlated with the tumor metastasis ability. However, cytoplasmic NDRG1 expression in lymph nodes with metastases was higher than in those without metastases, which is in agreement with earlier studies [18]. Other reports have supported the idea that NDRG1 is a metastasis

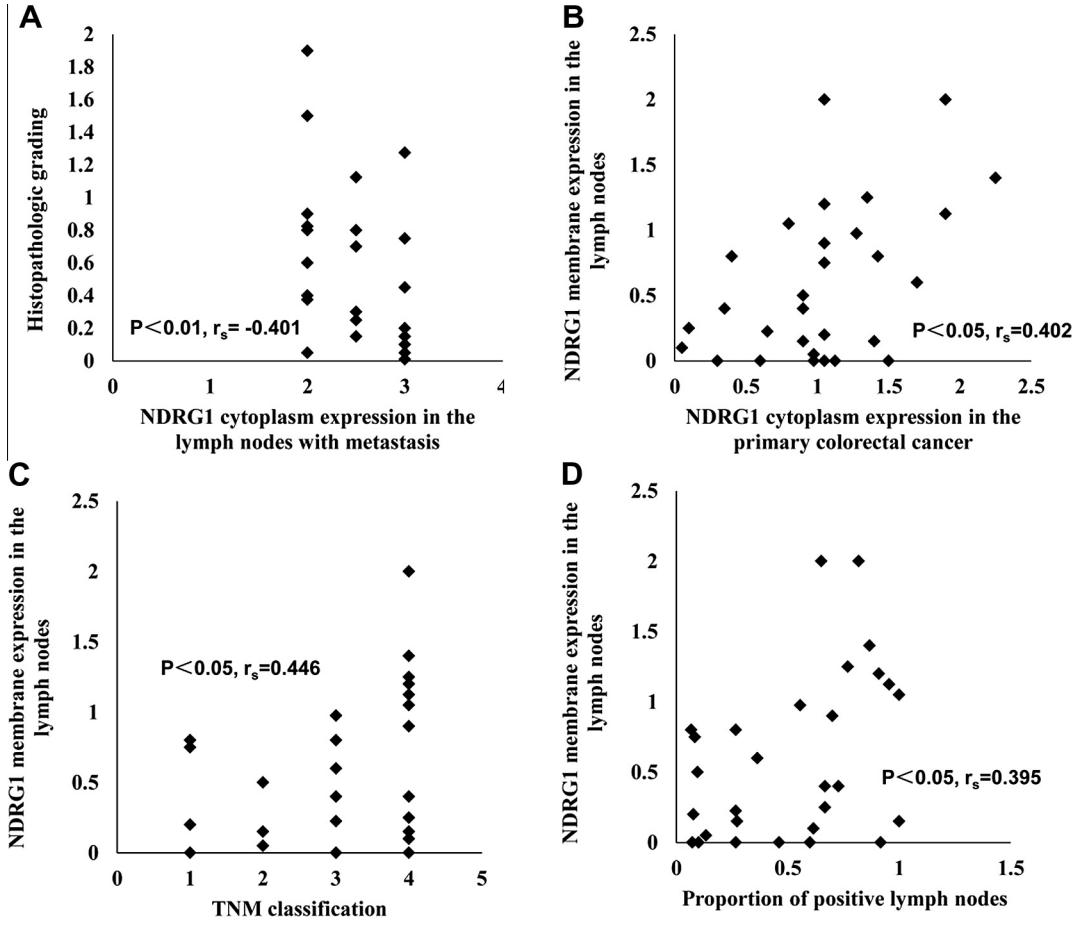

Fig. 3. Spearman's correlation coefficient analysis of the lymph nodes with metastasis group. (A) Negative correlation between lymph node staining and histopathological grading ($P < 0.01$; $r_s = -0.401$). (B) Positive correlation between cytoplasmic NDRG1 expression in the colorectal cancer tissues and membrane expression in the lymph nodes ($P < 0.05$; $r_s = 0.402$). (C) Positive correlation between NDRG1 membrane expression in the lymph nodes and TNM classification ($P < 0.05$; $r_s = 0.446$). (D) Positive correlation between NDRG1 membrane expression in the lymph nodes and the proportion of positive lymph nodes ($P < 0.05$; $r_s = 0.395$).

suppressor gene. Therefore, a certain relationship may exist between the cytoplasm expression of NDRG1 in lymph nodes and the probability of lymph node metastasis. NDRG1 inhibited metastasis in colon cancer by preventing actin-filament polymerization and stress fiber assembly and formation in the cytoplasm [21]. Perhaps NDRG1 expression in the lymph nodes with metastases plays a role in inhibiting tumor cells from forming distant metastases. In the lymph nodes metastases group, higher cytoplasmic NDRG1 expression correlated with better differentiation. NDRG1 has been shown to induce morphological changes consistent with differentiation and to up-regulate the expression of several epithelial cell differentiation markers [22]. Therefore, NDRG1 may suppress metastasis by inducing colorectal cancer cell differentiation and by partially reversing the metastatic phenotype.

NDRG1 can maintain membrane E-cadherin and β-catenin and inhibit TGF-β-stimulated cell migration and invasion [23]. Our results showed that NDRG1 membrane expression was significantly lower in the primary colorectal tumors than in normal colorectal mucosa, which may indicate that membrane expressed NDRG1 might inhibit the development and progression of colorectal cancer. Our results also showed that there was a significantly higher membrane NDRG1 expression in the lymph nodes with metastases than in those without metastases. Furthermore, in the lymph nodes metastases group, NDRG1 membrane expression increased the possibility of lymph node metastases, and NDRG1 membrane expression was positively correlated with TNM classification and the proportion of positive lymph nodes. These results indicate that membrane NDRG1 expression in lymph nodes is involved in lymph nodes metastasis.

In the lymph nodes metastases group, nuclear NDRG1 expression was higher in the colorectal cancer tissues than in the normal colorectal mucosa. In fact, there was no nuclear NDRG1 expression in normal colorectal mucosa. However, in the lymph nodes metastasis group, the membrane expression was lower in the colorectal cancer tissues than in the normal colorectal mucosa. Thus, we propose that NDRG1 expression may translocate from the membrane of the colorectal cancer tissue into the nucleus where it is involved in lymph nodes metastasis. However, few studies have focused on nuclear NDRG1 expression. Prior studies have reported that NDRG1 could translocate to the nucleus after DNA damage [24], and these data indicate that NDRG1 might be involved in DNA repair and proper DNA replication.

In summary, it appears that the differences in NDRG1 subcellular localization in normal colorectal mucosa, colorectal cancer tissues, and corresponding lymph nodes may affect the function of NDRG1 protein and may determine the probability of lymph nodes metastasis. For an individual patient, a combined analysis of NDRG1 subcellular expression in cancerous, non-cancerous, and corresponding lymph node tissues as well as the clinical variables will help predict the incidence of lymph node metastasis. The detection and analysis of NDRG1 subcellular localization in a larger sample group will further validate our conclusions and clarify the role of NDRG1 subcellular localization in predicting the occurrence of lymph nodes metastasis.

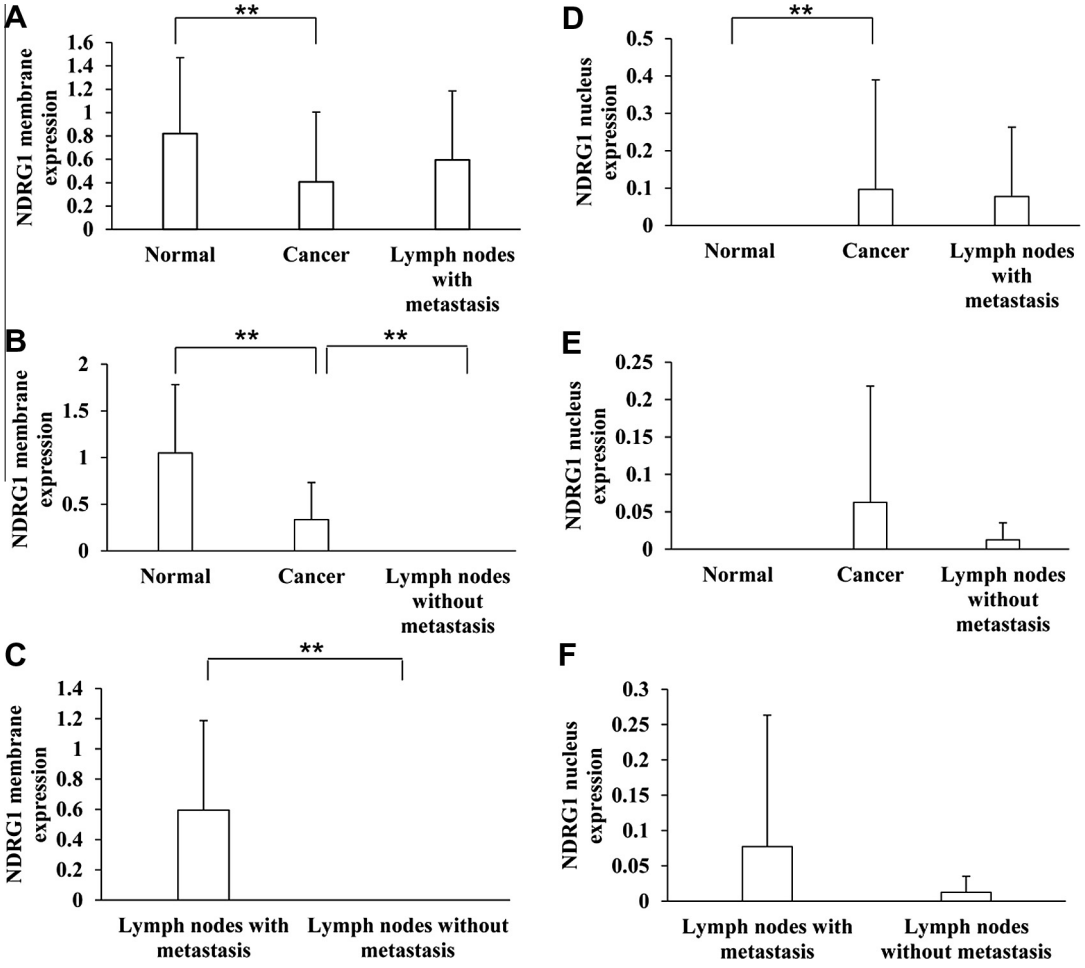

Fig. 4. NDRG1 membrane and nuclear expression in the different groups. (A) NDRG1 expression was higher in the normal mucosa than in the lymph nodes with metastases ($P < 0.01$). (B) NDRG1 expression was higher in the normal mucosa than in the colorectal cancer in the group of lymph nodes with metastases ($P < 0.01$) and NDRG1 expression was higher in the colorectal cancer than that in the lymph nodes without metastases ($P < 0.01$). (C) NDRG1 expression in the lymph nodes with metastases was higher than in the lymph nodes without metastases ($P < 0.01$). (D) NDRG1 expression in the normal mucosa was lower than that in the colorectal cancer in lymph nodes metastasis group ($P < 0.01$). (E) No significant difference was observed in lymph nodes without metastasis group. (F) No significant difference was observed between lymph nodes with and without metastases. $^{*}P$ value <0.05, $^{**}P$ value <0.01.

Acknowledgments

This work was supported by grants from the Natural Science Foundation of China (30873025) and from the Natural Science Foundation of Shandong Province (ZR2010HM033).

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