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The Regulatory Role of Colorectal Adenocarcinoma Hypoxia-Inducible Factor-1α in the Expression of N-myc Down-Stream Regulative Gene 1

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Abstract: HIF-1α participates in the neovascularization of colorectal adenocarcinoma tumor and the development of tumor via regulating VEGF expression. NDRG1 was over expressed in the process of colorectal adenocarcinoma. Current studies demonstrate that hypoxia is a NDRG1-inducible factor and HIF-1α also has taken part in this process. The study was to investigate regulation relationship between HIF-1α and NDRG1 in the LS174T cells and the two proteins associated with human colorectal carcinoma Duke classification. Construct siRNA expressive carrier pSilence-2.1-U6-siRNA targeting Hypoxia-Inducible Factor-1α (HIF-1α), hypoxially cultured LS174T cells were transferred by liposome. HIF-1α and NDRG1 mRNA and protein expression level was analyzed by RT-PCR and Western blot. In addition, 62 cases of clinical tissue specimen of human colorectal carcinoma were collection to detect HIF-1 and NDRG1 expression level using ISH, IHC. HIF-1α gene expression was inhibited by siRNA at transcriptional and protein level in the LS174T cells under hypoxia conditions for 24 h. Meanwhile, NDRG1mRNA expression was also significantly suppressed. In colorectal adenocarcinoma organization, HIF-1α and NDRG1 positive expression was both significantly correlated with Dukes staging ($p<0.05$). Moreover, spearman correlation analysis displayed HIF-1α was correlated with NDRG1 ($p<0.05$). The over expression of HIF-1α and NDRG1 likely play pivotal roles in the development of colorectal gland adenoma canceration and colorectal adenocarcinoma.

Key words: Hypoxia-inducible factor-1α, N-myc down-stream regulative gene 1, small interferring RNA, colorectal adenocarcinoma, China

INTRODUCTION

Current study demonstrates Hypoxia-Inducible Factor-1 (HIF-1) plays a central regulative role in promoting the infiltration and metastasis mechanisms of tumor cells (Jiang *et al*., 2004). The HIF-1 is constituted of two sub-units that is HIF-1α and HIF-1β. The HIF-1α is the sole O_2 regulating sub-unit, it determines the activity of HIF-1. Earlier studies indicated that HIF-1α participates in the neovascularization of colorectal adenocarcinoma tumor and the development of tumor via regulating VEGF expression (Jiang *et al*., 2004).

NDRG1 (N-myc downstream regulated gene 1, namely Cap43, Drg 1, RTP or rit42), a kind of stress-responsive protein, it has different designation due to different labs discovered it one after another. NDRG1 is a member of N-myc down-stream regulating gene family. Studies indicated that in the process of colorectal adenocarcinoma, NDRG1 was over expressed. Taken as a possible candidate tumor metastasis gene *NDRG1* can predict colorectal adenocarcinoma metastasis at an early time (Wang *et al*., 2004).

Current studies demonstrate that hypoxia is a NDRG1-inducible factor, HIF-1α also has taken part in this process (Cangul *et al*., 2002). Local organization hypoxia may occur in the process of fast growth of solid tumor. Researchers shall observe the regulative role of colon carcinoma LS174T cell and glands of large intestine cancer organization HIF-1α on NDRG1 and the meaningful function in tumor progress.

MATERIALS AND METHODS

LS174 T cell line was a kind gift from Dr. Shiquan Liu in Zhongnan Hospital at Wuhan University. DH5a competent cells were maintained and kept in the lab. The pSilence-2.1-U6 plasmid was purchased from Ambion. Restriction enzymes used in this study, r-taq and ex-taq

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were purchased from TaKara Inc. T4 DNA ligase from Promega; Rnasin RNAase inhibitor from Huamei Inc.; M-MLV reverse transcriptase and TRIZOL reagent from Invitrogen; Sofast™ transfection kit from Xiamen Taiyangma Biotech Inc.; high pure plasmid isolation kit from Shanghai Shenlenbochai Biotech Inc. *In situ* hybridization kit of HIF-1αmRNA was purchased from Wuhan Boster Biological Project Co.; polyclonal NDRG1 antibody (N-19) from Santa Cruz; polyclonal HIF-1α antibody from Santa Cruz.

Plasmid construction: According to human HIF-1α cDNA sequence in the GenBank (NM001530), targets for siRNA against HIF-1α mRNA were selected as described (Zhang *et al*., 2004; Sowter *et al*., 2003; Berchner-Pfannschmidt *et al*., 2004). The RNA oligos in sense and anti-sense directions corresponding to human HIF-1α siRNA1 at nucleotides 1213-1231 bp (sense: 5'-AGAGGUGGAUAUGUGUGGG-3'; anti-sense: 5'-CCCACACAUAUCCACCUCU-3'), siRNA2 at nucleotides 661-679 bp (sense: 5'- ACACACUGUGUC CAGUUAG -3'; anti-sense: 5'- CUAACUGGACACAGU GUGU -3') and siRNA3 at nucleotides 1540-1558 bp (sense: 5'-CUGAUGACCAGCAACUUGA-3'; anti-sense: 5'-UCA AGUUGCUGGUCAUCAG-3') with dTdT overhangs at each 3' terminus (Invitrogen).

To construct siRNA expression vector, two 64N primers each primer containing a 19N target sequence in the sense and antisense forms from the three different regions of HIF-1α were synthesized (Invitrogen). The sequences of target sites in the primers were underlined and the sequences of primers were:

HIF-1α siRNA1
sense: 5'-GATCCGAGAGGTGGATATGTGTGGGTTCAAGAGACCAC ACATATCCACCTCTTTTTTTGGAAA-3';
anti-sense:5'-AGCTTTTCCAAAAAAAGAGGTGGATATGTGTGG GTCTCTTGAACCCACACATATCCACCTCTCG-3';
HIF-1α siRNA2
sense: 5'-GATCCGCTAACTGGACACAGTGTGTTTCAAGAGAACAC ACTGTGTCCAGTTAGTTTTTTGGAAA-3';
anti-sense:5'-AGCTTTTCCAAAAAACTAACTGGACACAGTGTGTTC TCAAACACACTGTGTCCAGTTA GCGT-3';
HIF-1α siRNA3
sense: 5'-GATCCGCTGATGACCAGCAACTTGATTCAAGAGATCAA GTTGCTGGTCATCAGTTTTTTGGAA-3';
anti-sense:5'-AGCTTTTCCAAAAAACTGATGACCAGCAACTTGAT CTCTTGAATCAAGTTGCTGGTCATCAGCG-3'.

Sense and antisense primers were then cloned into pSilence-2.1-U6 plasmid at BamHI and HindIII sites according to the manufacturer instructions after annealing. Plasmids containing the right insert were confirmed by sequencing. A control plasmid containing an insertion of unrelated sequence was used as control.

Cell culture and transfection: LS174T cells were maintained in RPMI 1640 (GIBCO/BRL) supplemented with 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10 g L^{-1} heat-inactivated fetal bovine serum at 37°C under 5% CO_2. Cells were seeded onto 24 well plates at a density of 1.0×10^5 or 4.0×10^5 cells per 24-well plate or 6 well plate and grown to the confluence reaching approximately 60% at the time of transfection. The cells were transfected with 0.45 or 1.2 μg pSliencer-2.1-U6-siRNA using Sofast™ transfection reagent. Hypoxia conditions were reported earlier (Jiang *et al*., 2004).

RT-PCR: RT-PCR was performed to measure transcripts of HIF-1α and NDRG1 using that of beta-actin as an invariable control. Primers used in this study are listed as follow:

HIF-1α upstream 5'-GCATCTCCATCTCCTACCCACA-3',
downstream 5'-TGCATCCTTTTACACGTTTCCA-3' (358 bp);
NDRG1 upstream 5'-CGGCTGCCAGGTTCCTGTA-3',
downstream 5'-CCCGATCCCCGACTTTTCTA-3' (254 bp);
β-actin upstream 5'-CTTGCCATCCTAAAAGCCACC-3',
downstream 5'-GACCAAAAGCCTTCATACATCTC-3' (231 bp)

The base pair numbers in parenthesis represent PCR products of each gene after amplified by each pair of primers.

Western blot: Western blots for HIF-1α and NDRG1 was performed as described briefly as followed. A primary goat polyclonal NDRG1 (N-19) and HIF-1α antibody (Santa Cruz Biotechnology, Inc.) was used according to the manufacturer's protocol. Cells were harvested at the indicated time points, washed once with cold PBS and lysed in lysis buffer (PBS, 0.01 g L^{-1} EDTA, 1 g L^{-1} Triton) containing cocktail (Roche, USA). About 50 μg whole-cell extracts per lane were resolved using SDS-PAGE. The proteins were then transferred onto nylon membranes in the blotting buffer (5 mL L^{-1} [vol./vol.] methanol, 25 mmol L^{-1} Tris, 120 mmol L^{-1} glycine). Membranes were blocked with 5 g L^{-1} nonfat dried milk, 2 g L^{-1} bovin serum albumin and TBS-T. Endogenous NDRG1 was detected with 1:600 dilution of anti-NDRG1 and anti-HIF-1α polyclonal antibody followed by a secondary rabbit anti-goat IgG antibody. The bands were visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

Patients and tumor specimens: Tissue specimens used in this study included 18 specimens of colorectal adenomas (patient mean age 49 years, ranging from 30-79 years) and 62 colorectal adenocarcinomas (patient mean age 52 years,

ranging from 25-89 years) were obtained from the Department of Pathology, Zhongnan Hospital, Wuhan University, Wuhan, China. All specimens were resected surgically or endoscopically and the diagnoses were confirmed pathologically. No patients had undergone preoperative radiotherapy or chemotherapy. About 62 specimens were classified according to level of differentiation: 17 cases were well differentiated, 30 were moderately well differentiated and 15 were poorly differentiated. The tumors were classified into 4 stages according to the Dukes' System as follows: Dukes A (17 cases), Dukes B (18 cases), Dukes C (20 cases) and Dukes D (7 cases). The clinical features of all patients including stage, histological type, synchronous liver metastasis and lymph node metastasis were obtained from clinical and pathological reports. In addition, 18 specimens of colorectal adenoma were included in this study (10 cases of tubulovillous adenoma, 4 of tubular adenoma and 4 of villous adenoma). All resected tissue specimens were fixed by 40 g L^{-1} formalin after removal from the patients, embedded in paraffin and cut into 4 μm serial sections.

***In Situ* Hybridization (ISH) of HIF-1α and NDRG1 mRNA:** Hybridization was conducted according to the previous study (Jiang *et al*., 2004). In tissue specimens, positive hybridization signal was defined as small brow granules in nucleus or cytoplasma under the microscope at 400 fold magnification. Five fields were chosen randomly in each slide. About 200 cells were counted in each field. Total 1000 cells from five fields were used to calculate the positive rate. If the HIF-1α positive rate is <1%, it was considered negative for HIF-1α mRNA. If the positive rate is bigger or equal to 1%, it was considered as positive for HIF-1α mRNA. For HIF-1α mRNA, positive rate <5% (-), 6-25% (+), 26-50% (++), 51-75% (+++) and >76% (++++). Meanwhile, in LS174T cells, the average optical density and the rate of positive area of expression of HIF-1α and NDRG1 mRNA were analyzed using image analysis Image HPIAS-2000 Analysis System.

Immunohistochemistry of HIF-1α and NDRG1 protein: The procedures for immunohistochemical detection were performed according to the earlier study (Jiang *et al*., 2004). For NDRG1, if the positive rate is <1%, it was considered negative for HIF-1α mRNA. If the positive rate is bigger or equal to 1%, it was considered as positive for NDRG1. The protein positive rate <5% (-), 6-25% (+), 26-50% (++) and >50% (+++). All above also applies to the standard classification HIF-1α in tissue specimens.

Meanwhile, the average optical density and the rate of positive area of expression of HIF-1α and NDRG1 protein in LS174T cells were analyzed using image analysis Image HPIAS-2000 Analysis System.

Statistical methods: SPSS11.5 was used as the software for statistical analysis.

RESULTS AND DISCUSSION

HIF-1α siRNA3 at nucleotides 1540-1588 bp was chose as an effective inhibition to HIF-1α expression: To select the most effective inhibition of HIF-1α siRNA fragment, the three siRNA expression vectors were applied to LS174T cells. The siRNA-HIF-1α1540-1558 insert effectively inhibited *HIF-1α* gene transcription which resulted that the mRNA expression level of HIF-1α detection by RT-PCR was reduced 67.61% ($p<0.05$) and the HIF-1α protein expression level detecting by Western blot was reduced 53.53% ($p<0.05$). Whereas pSliencer-2.1-U6-siRNA-HIF-1α1213-1231 and pSliencer-2.1-U6-siRNA-HIF-1α661-679 which suppression ratio separately were 3.18 and 5.46%, revealed no inhibitory effect compared with nc group ($p>0.05$). Furthermore, researchers found that in LS174T cells, transfected by pSliencer-2.1-U6-siRNA-HIF-1α1540-1558, both mRNA and protein expression levels of NDRG1 were inhibited. Compared with nc group, NDRG1 mRNA expression levels was decreased 26.81% and the protein expression level was decreased 91.78%. However, the other siRNA-HIF-1α expression vectors inhibition NDRG1 protein expression level revealed no inhibitory effect compared with nc group ($p>0.05$). Consequently, pSliencer-2.1-U6-siRNA-HIF-1α1540-1558 was chose for the following experiment to sufficiently knockdown HIF-1α (Fig. 1).

Expression level of HIF-1α and NDRG1 in LS174T cell and regulation by hypoxia: HIF-1α mRNA was expressed in LS174T cells under normoxic condition analyzed by ISH. Exposed to the hypoxic environment, significant increase in HIF-1α mRNA transcript was observed ($p<0.05$, Fig. 2a and c). Meanwhile, immunohistochemical detection indicated that HIF-1α and NDRG1 protein showed basal level expression in LS174T cells under normoxic conditions. Hypoxia induced a rapid and sustained accumulation of HIF-1α and NDRG1 protein in LS174T cells up to 24 h ($p<0.05$, Fig. 2b and d).

Inhibition of HIF-1α was sufficient to depress *NDRG1* gene transcription as well as translation: To test if the

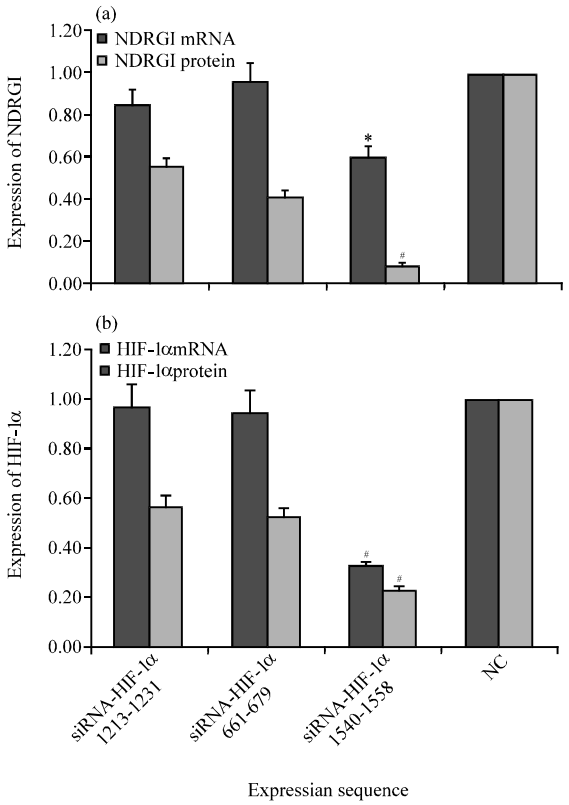

Fig. 1: pSliencer-2.1-U6-siRNA-HIF-1α decreased HIF-1α and NDRG1 expression in LS174T cells. LS174T cells were intervened with three different siRNA expression cassette sequences of siRNA-HIF-1α1213-1231, siRNA-HIF-1α661-679, siRNA-HIF-1α1540-1558 or with a Negative Control (NC); a) RT-PCR analyses of HIF-1α in the HIF-1α siRNA-expressing cells compared to nc. Western Blotting analyses of HIF-1α protein extracts from LS174T cells treated by siRNA- HIF-1α or Negative Control (NC) at 24 h after transfection. β-actin was used as an internal control and b) RT-PCR and Western blot analyses NDRG1 mRNA and protein expression level. The processing methods were as well as HIF-1α. Results are presented as means±SD. of these independent experiments. *$p < 0.05$ vs. nc. #$p<0.01$ vs. nc

gene transcription and translation of NDRG1 was affected by the expression level of HIF-1α within LS174T cells, loss-of-function approach was applied. Exposed to the hypoxic environment, LS174T cells were transfected with 0.45 or 1.2 μg pSliencer-2.1-U6-siRNA-HIF-1α1540-1558 after transfection 24 h, the mRNA expression of HIF-1α and NDRG1 was detected with *in situ* hybridization and the protein expression was measured by

Table 1: Colorectal gland adenoma and HIF-1α mRNA and NDRG1 protein expression with glands of large intestine cancer organization at different dukes stages

		HIF-1		NDRG1	
Expression	Cases	-	+	-	+
Adenomas	18	10	8	14	4
Adenocarcinoma	(n = 62)	(n = 20)	(n = 42)	(n = 28)	(n = 34)
Stage A	17	10	7	12	5
Stage B	18	5	13	8	10
Stage C + D	27	5	22	8	19

immunohistochemistry method. As well as the significant inhibition of HIF1α expression ($p<0.05$, Fig. 2c), the decreasing of mRNA expression of NDRG1 was identical with the result of RT-PCR test in the result. Meanwhile, the protein expression after transfection which was consistent with result of mRNA was inhibition compared with nc group ($p<0.05$, Fig. 2d). These data suggested that expression of NDRG1 was positively regulated by HIF-1α.

Relationship of HIF-1α, NDRG1 in colorectal adenocarcinoma organization: HIF-1α expression in colorectal gland adenoma and adenocarcinoma was assayed with ISH. HIF-1α mRNA positive reaction products were hybridizational buffy grains and were mainly located at tumor cellular endochylema. HIF-1α mRNA positive expressive rate (42/62) in adenocarcinoma group was significantly higher than adenoma group (8/18, $p<0.05$). In the evolution process from Dukes A stage to C+D stage, significant differences were shown in the HIF-1α mRNA positive rates of the slices of each stage (Table 1). Additionally, NDRG1 positive expressive rate (34/62) in adenocarcinoma group was significantly higher than adenoma group (4/18, $p<0.05$). NDRG1 positive expression is significantly correlated with Dukes staging (Dukes A 5/17, Dukes B 10/18, Dukes C+D19/27, $p<0.05$). Moreover, Spearman correlation analysis displayed that HIF-1α was positively correlated with NDRG1 ($rs = 0.675$, $p<0.05$) (Table 2).

NDRG1 is a member of N-myc down regulation genetic lineage. It is first discovered and isolated from colon cancer cell line HT-29 D4 by a Dutch researcher Van Belzen using modified differential display method in 1997 (Van Belzen *et al*., 1997). Due to the initial study of Van Belzen showed that this gene is associated with cell differentiation so it was designated as differentiation related gene1 and Drg1. Later, this gene was formally denominated NDRG1 by Human Genome Organization Gene Designation Committee. *NDRG1* gene is located at human chromatin 8q24.3 with a relative molecular weight of 43000, approximately 60 kb of length including 16 exons and 15 introns. NDRG1 extensively participates in many kinds of biological functions like growth and

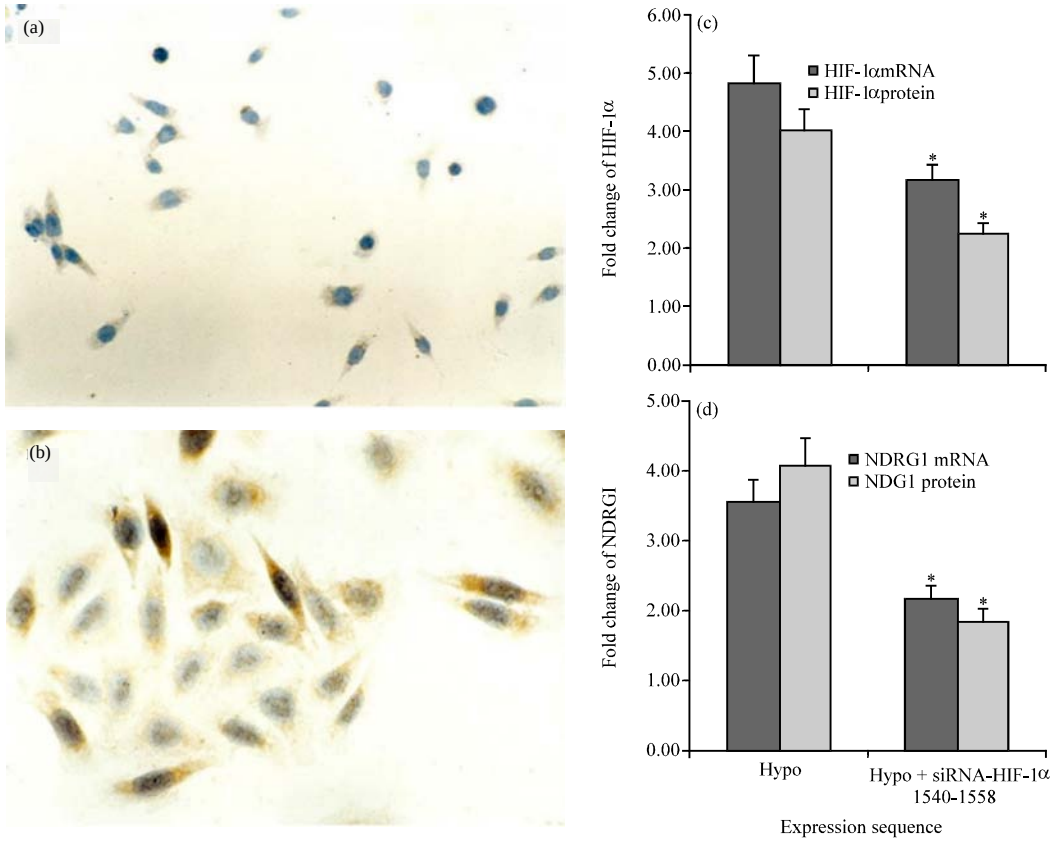

Fig. 2: HIF-1α and NDRG1 mRNA and protein expression were detected under hypoxia (Hypo) or together with pSliencer-2.1-U6-siRNA-HIF-1α1540-1558 trasfection (Hypo+siRNA-HIF-1α1540-1558). Results are presented as means±SD of these independent experiments. *p<0.05 vs. Hypo; a) Positive expression of HIF-1αmRNA under hypoxia by ISH x200; b) Positive expression of HIF-1α protein in endochlema under hypoxia by IHC S-P test x400; c) Fold change of HIF-1α; d) Fold change of NDRGI

Table 2: Correlation of HIF-1α expression in colorectal adenocarcinoma organization with NDRG1

		Expression of HIF-1α				
Expression of NDRG1	Cases (n = 62)	-(n = 20)	+(n = 12)	++(n = 22)	+++(n = 8)	p-value
-	28	18	4	4	2	<0.05
+	8	1	3	3	1	-
++	19	1	3	13	2	-
+++	7	0	2	2	3	-

development of organizational cells, induction of tumor cell differentiation, participation in stress reactions and etc., many researchers have done a significant number of studies on the relationship between it and cell differentiation.

NDRG1 gene was initially discovered in the study of Nickel compound exposure-inducible carcinogenesis etiology. NDRG1 genetic transcription and expression effect significantly in Nickel compound-inducible carcinogensis and it switches on the occurrence of tumor. Presently, little study has done on the relations between HIF-1α and NDRG1. There is a HIF-1 binding site at NDRG1 promoter, there are two HIF-1 binding sites at 3'non-typing zone (Wang *et al*., 2004). NDRG1 is a *hypoxia-inducible* gene, hypoxia may significantly induce cell NDRG1 expression but this effect requires the cooperation of nuclear factor HIF-1. Hypoxia Induces HIF-1+/+ murine fibroblast to express NDRG1 whereas HIF-1-/-murine fibroblast was not expressed. Results indicated, hypoxia-inducible NDRG1 genetic expression requires nuclear factor HIF-1 (Salnikow *et al*., 2003). Recently, Han *et al*. (2006) found from studies that the expression of 2 mL L^{-1} O_2 and $CoCl_2$-treated human leukemia U937 cell HIF-1alpha and NDRG1 was all significantly elevated. Results of this study displayed, targeting small interfering RNA significantly suppressed the expression of HIF-1α mRNA, the expression of NDRG1 mRNA and protein was also significantly degraded which confirmed the positive regulatory role of HIF-1α on NDRG1 expression. Thus, researchers presume in colon cancer cell line, hypoxia can induce NDRG1 expression via HIF-1α.

Currently, studies initially displayed that NDRG1 possibly has taken part in the infiltration and metastasis of malignant tumor. NDRG1 was over expressed in the tumor organization of pulmonary carcinoma, liver cancer, breast cancer, brain cancer, kidney cancer, melanoma, etc. (Cangul, 2004). Chang had reported the state of NDRG1 expression in the tumor organization of 52 cases' Oral Squamous Cell Carcinoma (OSCC) patients: RT-PCR displayed tumor organization NDRG1 mRNA is 12.9 fold normal epithelial organization; Western blot also displayed that tumor organization NDRG1 protei (with lymphatic and hepatic metastasis) at Dukes C, D stage was over expressed compared to the patients of A, B stage and it was closely correlated with the poorly differentiation of tumor (Chang *et al*., 2005). Shah reported 131 cases of metastasis colon cancer, expression of NDRG1 was found in all of the liver metastasis specimens, this study demonstrated that NDRG1 is perhaps a tumor metastasis gene (Shah *et al*., 2005). Researchers found from the experiment that the expression of NDRG1 protein at Dukes C, D stage (with lymphatic gland, liver metastasis) was significantly higher than that of A, B stage patients indicating elevated NDRG1 genetic expression is capable of promoting lymphatic gland and liver metastasis of colorectal adenocarcinoma. Meanwhile, both expression was positively correlated and was intimately related with the progress of tumor. Current studies also have confirmed that HIF-1α plays pivotal roles in the progress of colorectal adenocarcinoma (Shah *et al*., 2005).

CONCLUSION

In this study, HIF-1α is correlated positively with NDRG1 expression in colorectal adenocarcinoma organization indicating NDRG1 is likely another molecular mechanism for the participation of HIF-1α in the infiltration and metastasis of colorectal adenocarcinoma.

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