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Identification of differential proteins in colon cancer SW480 cells with HIF1-α silence by proteome analysis

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Hypoxia is one of the basic characteristics of the colorectal cancer and HIF1 plays a central role in tumor hypoxia adaptation and controls the expression of a variety of genes. But it had not reached a comprehensive understanding of them in colorectal cancer in hypoxia. This study silenced HIF1-α, which is the regulatory subunit of HIF1, and established two dimensional gel electrophoresis profiles for human SW480siHIF1-α and SW480N cells to identified proteins with differential expressions. Ten down-regulated proteins and nine up-regulated proteins were identified by HPLC-Chip-MS/MS. Down-regulated proteins including PLD2, ANXA2. HIF1-α had a clear positive correlation with PLD2 and ANXA2 in colon adenocarcinoma samples. There proteins might have a direct or indirect contact HIF1-α, and play a critical role in apoptosis, signal transduction and hypoxia adaptation.

Key words: colorectal neoplasms; hypoxia inducible factor1-α; RNA interference; proteomics analysis; phospholipase D2; annexin A2

Colorectal cancer (CRC) is the fourth most common cancer type worldwide. In Asia, CRC has emerged as the second most common cancer [1]. Although therapeutic innovations and increasing education on lifestyle to prevent colorectal cancer, there are approximately 148,800 new cases and 50,000 deaths each year in US [2]. Hypoxia induces a number of changes in gene expression in tumor cells [3]. It is considered as one of the tumor microenvironment features favoring tumor cell survival [4], suppresses the differentiation and apoptosis, and induces multidrug resistance [5]. The adaptive response to hypoxia is adjusted by a family of transcription factors. The most important member of this family is hypoxia inducible factor1 (HIF1). It is a heterodimer consisting of a HIF1-α subunit and a HIF1-β subunit. HIF1-β subunit are constitutively expressed, but the formation of HIF1 transcription factor in the nucleus depends on HIF1-α stabilization, which is mainly O_2-dependent [4].Since the discovery in the early 1990s, HIF1 has been rapidly attracted interest for its involvement in tumor metabolism, angiogenesis and metastasis, and its potential role as cancer therapeutic target [6]. Moreover, it has recently been reported, by using DNA microarrays, that more than 2% of all human genes are regulated by HIF1 in arterial endothelial cells, directly or indirectly [7]. HIF1 plays complicated roles in cancer biology, but there is still a need for understanding many of them. Further verification which are the HIF1 downstream genes and their functions in CRC in hypoxia is being searched.

Materials and methods

Construction of HIF1-α shRNA expression plasmid and stable transfection. Two pair of shRNA targeting HIF1-α (GeneBank accession NM_001530) were constructed and subcloned into pGenesil-11 plasmid. The sequence was 5'-caaaaa CTA ACT GGA CAC AGT GTG T ttcg ACA CAC TGT GTC CAG TTA GC ggtgtttcgtc-3' and 5'-gaaaaa AGC CAC TTC GAA GTA GTG C tctc GCA CTA CTT CGA AGT GGC T gggaaagagtg-3'. All of the sequences were not homologous with any human coding gene by BLAST analysis. The shRNA was constructed into pGenesil-11 plasmid by Genesil Biotechnology Company (Wuhan, China) and a black plasmid served as a negative control, namely, SW480siHIF1-α and SW480N respectively. In blank control group, PBS was in place of plasmid. Cells were transfected with Lipofectin2000 (Invitrogen, USA) according to the manufacturer's protocol.

Cell lines and cell culture. Human colon adenocacinoma cell line SW480 was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured

in RPMI Media 1640 (GIBICO, USA) supplemented with 10% (vol/vol) fetal bovine serum (GIBICO, USA) at 37°C in a humidified incubator containing 5% CO_2. To reach and control hypoxia conditions, cells were placed in a three-chamber air incubator (Thermo, USA) and flushed with a gas mixture of 5% CO_2-95% N_2 at 37°C; the final medium pO_2 value was consistently measured below the 0.5% to 1% range. We chose to explore the function of HIF1-α on colon adenocacinoma cell by using a scheme protocol of 1h hypoxia interrupted by 30min periods of reoxygenation.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared from cells using the RNAiso reagent (TAKARA, China) on days 3, 7 and 15 as recommended by manufacturer. RT-PCR was used for the analysis of HIF1-α mRNA with GAPDH as an internal control. Primers for HIF1-α were as follow: forward primer 5'-AGA AAC CAC CTA TGA CCT GC-3', reverse primer 5'-TGA GTT TCA ACC CAG ACA TA-3'. The GAPDH primers were forward 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse 5'-TCC ACC ACC CTG TTG CTG TA-3'. Reactions were performed in accordance with the standard protocol. One step RT-PCR was performed at 50°C for 30 min, 2 min at 94 °C; followed by 27 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. The products were separated by electrophoresis in 3% agarose and visualized with ethidium bromide.

Western blotting analysis. Cells from each group were harvested and the protein extracts were separated via sodium dodecyl sulfate-polyacdene gel electrophoresis and transferred onto nitrocellulose membranes. The membrane was then blocked with the primary polyclonal antibodies against HIF1-α, Phospholipase D2 (PLD2), Annexin A2 (ANXA2) and β-actin (rabbit anti-human, Abnova, Taiwan) at 4°C overnight. After washing with TBS containing 0.05% Tween20 the membranes were incubated with secondary antibody (goat anti-rabbit, Santa Cruz, USA) for 2 h. They were visualized by chemiluminescence system according to manufacturer's instruction.

Protein sample preparation. We detected the HIF1-αmRNA level in transfected cells by RT-PCR and confirmed by Western Blotting. So we knew when were the best interference effects and these day cell proteins were harvested for proteomics research. Lysed in buffer (7 M urea, 2M thiourea, 65 mM DTT, 4% Chaps, 0.2% Bio-Lyte, 20ug/mlDNase and 5ug/mlRNase). After incubation on ice for 1 h, the lysates were centrifuged at 12000×g for 30 min at 4°C. The supernatant was transferred and packaged separately, and then stored at -80°C until use. The concentration of the total proteins was determined using protein quantification kit.

Two dimensional gel electrophoresis. 200ug protein samples were diluted to 500ul with rehydration solution and applied to IPG strips (pH 3–10L) by 16h rehydration. Then strips were focused to give a total of 50,000Vh on Bio-Rad PROTEAN IEF Cell. Equilibrated IPG strips were performed on Bio-Rad PROTEAN II xi Cell vertical electrophoresis bath system. After SDS–PAGE, the gels were stained by Bio-Rad silver stain kit (Bio-Rad, USA) as recommended by manufacturer.

Gel scanning and image analysis. Image acquisition and analysis were performed with GS800 Calibrated Densitometer Scanner (Bio-Rad, USA) and Quantity One 4.5.0 Scanning software (Bio-Rad, USA). Comparisons were made between gel images of SW480siHIF1-α and SW480N cell pair by pair. Proteins were classified as being differentially expressed between the two cell lines when spot intensity was showed a difference ≥ 2-fold. Consistently and significantly spots were selected for analysis by HPLC-CHIP-MS/MS.

Protein identification by HPLC-Chip-MS/MS. Selected differential protein spots were excised from preparative gels. Pretreatment of protein spots and HPLC-Chip-MS/MS conditions (Agilent, USA) were according to manufacturer's instruction. At last, the data were processed by Spectrum Mill, identified in NCBInr database.

Immunohistochemistry of tumor sections. Twenty colon adenocarcinoma samples were obtained from the department of Gastrointestinal Surgery in the First Affiliated Hospital of Chongqing Medical University, during the years 2009.2–2009.4. Tumors were fixed in 4% paraformaldehyde and were processed through a series of increasing ethanol concentrations for paraffin embedding. 4 μm sections were obtained, then deparaffinized in xylene, rehydrated and treated with 3% hydrogen peroxide for 20min and incubated with blocking serum (goat serum) at room temperature for 30min. Immunohitstochemistry was carried out by incubating sections with primary polyclonal antibodies against HIF1-α, PLD2 and ANXA2 (rabbit anti-human, Abnova, Taiwan), then with secondary antibody. The expressions of HIF1-α, PLD2, ANXA2 were visualized by Chromogen 3, 3-diaminobenzidine immunolabeling. At last, the sections were counterstained with hematoxylin. It was defined positive if over 10% of cancer cells were immunoreactive.

Statistical analysis. One-way ANOVA, Spearman rank correlation were processed by the statistical software SPSS 10.1. Statistical significance was assumed when $p<0.05$.

Results

Inhibition of HIF1-α gene expression. The expression levels of HIF1-αmRNA were detected by RT-PCR on days 3, 7 and 15 in each group. The cells stably transfected with pGenesil-11 plasmid were eliminated the HIF1-α expression, especially on day 7. While the expression level of HIF1-αmRNA in SW480N cell line transfected with empty pGenesil-11 plasmid and in blank group did not change. The HIF1-α protein expression analyzed by Western blotting showed a result consistent with results from RT-PCR. (Figure 1).

Protein separation. The protein spots of the samples were mostly distributed in the gel with the pI in the range of 4–8 and molecular masses of 20-110kd. The differential expression proteins were sieved. Among them, twenty-three proteins were obviously changed (Figure 2).

Protein identification. Compared to those in SW480N cell line, thirteen down-regulated proteins and ten up-regulated proteins were selected for MS/MS analysis (Figure 3). By search-

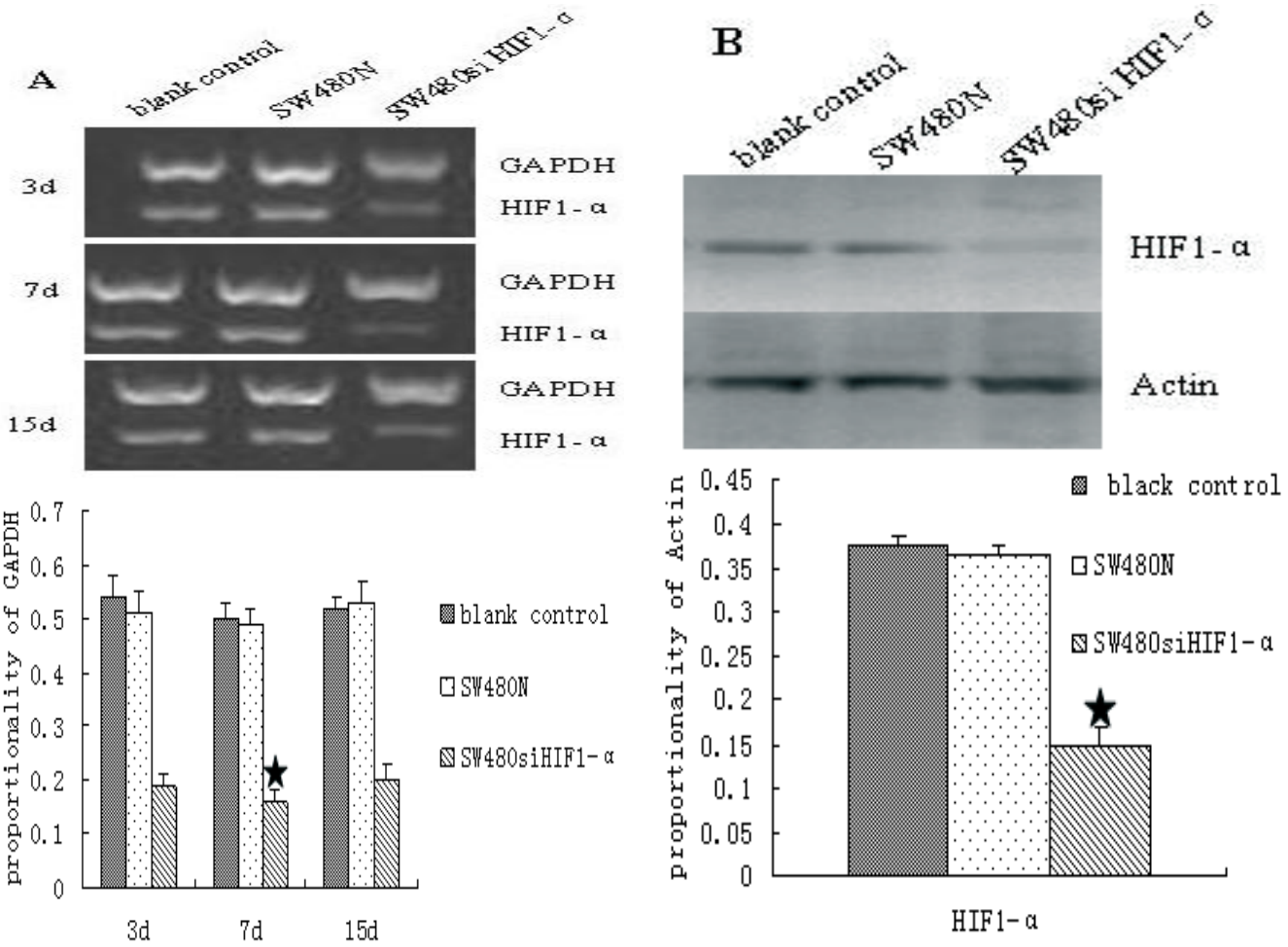

Figure 1. Stable suppression of HIF1-α expression in SW480 cells. (A) RT-PCR detection of HIF1-α mRNA expression in different times. The expression of HIF1-α mRNA suppressed in SW480siHIF1-α cells significantly, especially on day 7. The data were displayed as Mean ± S.D. *P<0.05 vs. other groups. (B) Western blot detection of HIF1-α protein expression on day 7. Stable expression of HIF1-α siRNA in SW480siHIF1-α induced a marked reduction in HIF1-α synthesis. The data were displayed as Mean ± S.D. *P<0.05 vs. SW480N or blank control group.

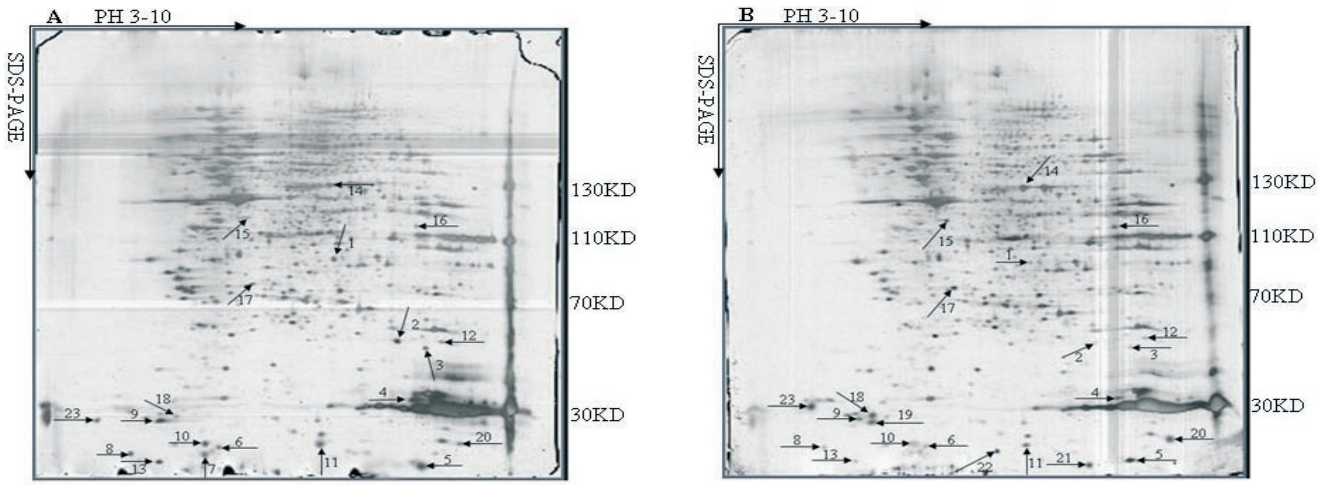

Figure 2. 2-DE maps of SW480N (A) and SW480siHIF1-α (B). Twenty-three differential expression proteins spots marked with arrows were identified using HPLC-CHIP-MS/MS.

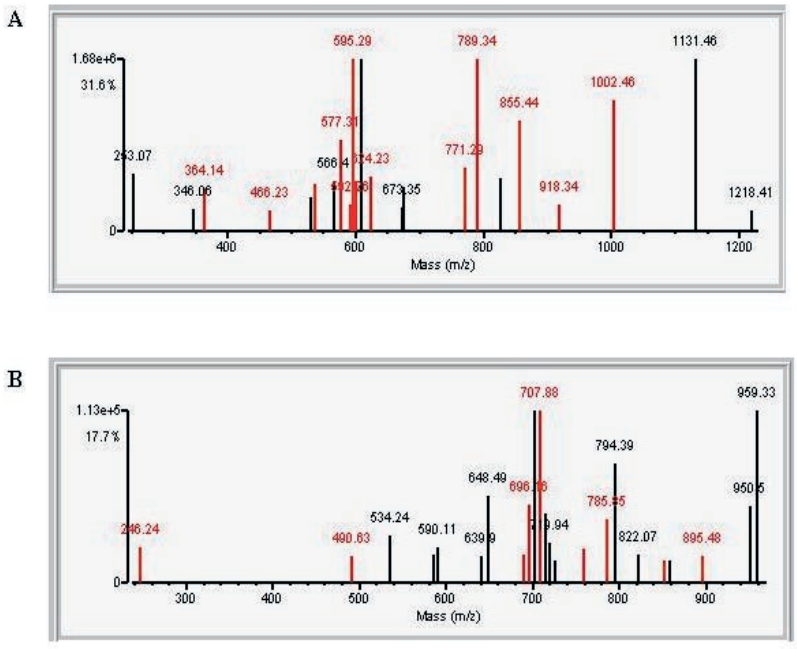

Figure 3. HPLC-CHIP-MS/MS analysis of differential expression protein spot 1 and 2. (A) The HPLC-CHIP-MS/MS mass spectrum of spot 1 identified as the PLD2 according to the matched peaks was shown. (B) The HPLC-CHIP-MS/MS mass spectrum of spot 2 identified as the ANXA2 according to the matched peaks was shown.

Table1. The information of differential proteins

Spot	Protein Name	PI	Protein MW (Da)	Mean Peptide Spectral Intensity	DatabaseAccession	Change
1	PLD2	7.41	105,987.7	8.93e+007	IPI00024727	down-regulated
2	ANXA2	8.53	40,411.4	1.09e+008	IPI00418169	down-regulated
3	GAPDH	8.76	38,791.5	3.31e+007	IPI00788737	down-regulated
4	MDH2	8.92	35,531.5	1.51e+007	IPI00291006	down-regulated
5	BTF3	9.40	22,168.1	2.88e+007	IPI00221035	down-regulated
6	SSR4	5.53	20,213.1	1.40e+007	IPI00019385	down-regulated
7	ATP5H	5.21	18,491.3	3.28e+007	IPI00220487	down-regulated
8	CALML5	4.34	15,920.6	1.86e+007	IPI00021536	down-regulated
9	TPM4	4.69	32,722.9	1.82e+008	IPI00216975	down-regulated
10	PRDX2	5.66	21,892.0	5.96e+006	IPI00027350	down-regulated
14	INPP5D	7.38	133,293.2	6.52e+007	IPI00329213	up-regulated
15	BUB1	6.03	122,375.9	7.81e+006	IPI00783305	up-regulated
16	SEMA6D	8.77	119,872.9	1.70e+008	IPI00178302	up-regulated
17	RFX2	6.29	80,003.7	7.88e+006	IPI00298182	up-regulated
18	TPM1	4.70	32,677.7	6.55e+007	IPI00000230	up-regulated
19	YWHAE	4.95	29,603.6	3.56e+007	IPI00086909	up-regulated
20	PTGDS	9.92	22,949.1	3.29e+006	IPI00513767	up-regulated
21	DSTN	8.06	18,505.9	4.36e+007	IPI00473014	up-regulated
22	ISG15	6.83	17,887.6	7.79e+007	IPI00375631	up-regulated

ing in NCBInr database using Spectrum Mill, nineteen proteins were identified, four kinds of protein failed to be identified. The information of the differential protein was listed in Table 1.

Expression confirmation by Western blotting. Western blotting was performed to verify two selected proteins, PLD2 and ANXA2. Figure 4 displays the gels of Western blotting,

confirming the decreased expressions of PLD2 and ANXA2 in SW480siHIF1-α cells.

Immunohistochemistry. HIF1-α, PLD2 and ANXA2 could be shown in the majority of our 20 colon adenocarcinoma samples. HIF1-α was present in 80.0% of cases, PLD2 and ANXA2 were present in 75.0% of cases. Three Proteins were expressed mainly in the cytoplasm, as shown in Figure 5 and HIF1-α had significant association with PLD2 (rs =0.504, $p<0.05$) and ANXA2 (rs =0.702, $p<0.01$).

Discussion

This study combined a proteomic approach with RNA interference technology to identify proteins associated with HIF1-α in SW480 cell line in hypoxia. We successfully used RNA interference technology to stably knock down HIF1-α gene expression in human colon cancer cell line SW480. As a reliable cell model, the well-resolved, reproducible 2-DE patterns of SW480siHIF1-α and SW480N cell line were established. Compared to those in SW480N cell, nineteen proteins were identified by HPLC-CHIP-MS/MS. Furthermore, HIF1-α had a clear correlation with PLD2 and ANXA2 in colon adenocarcinoma samples.

HIF1-α expression has been detected in the majority of solid tumors examined including brain, bladder, breast, colon, ovarian, pancreatic, renal, and prostate, whereas no expression was detected in surrounding normal tissue or in benign tumors. Up-regulation of HIF1-α increases HIF1 transcription factor activity and promotes tumor growth; whereas loss of HIF1 activity dramatically decreases angiogenesis, tumor growth and energy metabolism. Clinically, HIF1 overexpression has been indicated to be a biomarker of highly aggressive disease and associated with poor prognosis and treatment failure in

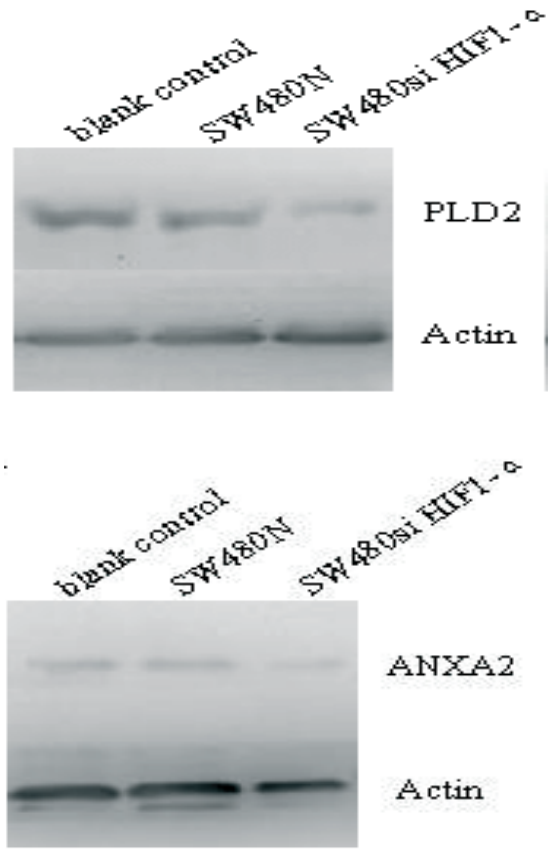

Figure 4. Inhibition of PLD2 and ANXA2 proteins expression in SW480siHIF1-α cells at the 7d. Treatment of cells with SW480siHIF1-α reduced PLD2 and ANXA2 proteins level, whereas proteins expression was not significantly suppressed in SW480N and blank control group.

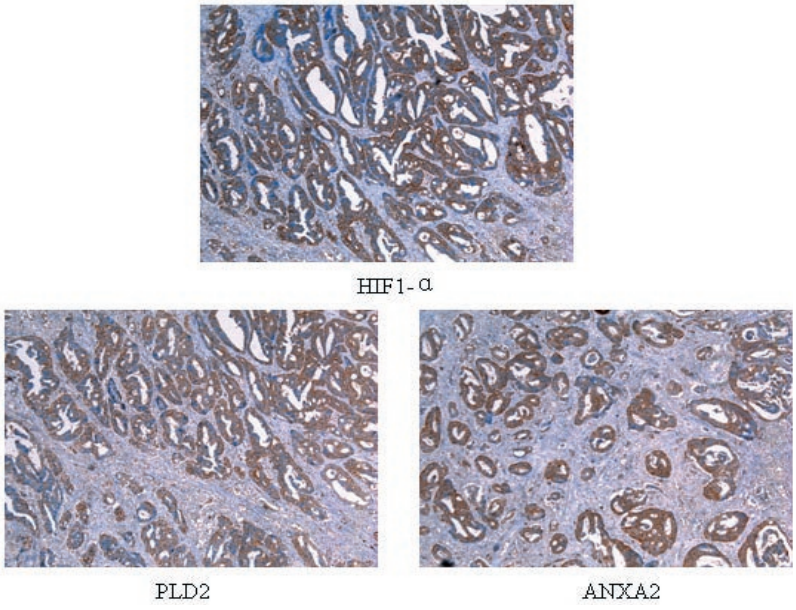

Figure 5. Detected expression of HIF1-α, PLD2 and ANXA2 proteins in colon adenocarcinoma samples by immunohistochemistry. Three proteins are mainly observed in the cytoplasm of the colon adenocarcinoma samples. (SP×200)

a number of cancers [8]. In this study there were twenty-three proteins obviously changed in SW480siHIF1-α cells, including PLD2 and ANXA2. They had critical role in apoptosis, signal transduction and hypoxia adaptation.

PLD2 is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid (PA) and choline [9]. Its cellular actions are correlated to the production of PA and include affection on cell growth, differentiation, survival and apoptosis. PA is a critical intracellular signaling molecule activating phospholipases, protein kinases and protein tyrosin kinases.

Cell migration is crucial during both physiologic as well as pathologic processes, . Silencing PLD2 gene expression with siRNA, result in a decrease in cell migration. PLD is known to be capable of modifying membrane architecture thought PA. It can regulate several key steps in membrane trafficking and cytoskeletal reorganization [10]. In addition, the activation of PLD2 may also mediate the antiapoptotic effect, and increased PLD2 activity in PC12 cells has been reported to reduce hypoxia-induced death [11]. Furthermore, the agonists of PLD2 improve myocardial functions and diminish the infarct size to ischemia, whereas the inhibition of PLD2 blocks the beneficial effects. Those prove that PLD2 is an important factor in tumor cells for hypoxia adaptation.

ANXA2 is a Ca^{2+}-dependent phospholipid-binding protein among the annexins family. It exists as two forms in the cells, a monomer and a heterotetramer (AIIt). The heterotetramer is composed of two subunits of ANXA2 linked together by a dimer of S100A10, an 11-kDa calmodulin- related protein, which is belong to the S100 protein family [12]. ANXA2 localized in the cytoplasm, nucleus, or membrane domains, which has been implicated in a number of membranerelated events, such as endocytosis and exocytosis, cell–cell adhesion, proliferation and cell surface fibrinolysis [13].

The expression of ANXA2 is induced in various transformed cells. Up-regulated ANXA2 has been reported in human hepatocellular carcinoma, pancreatic adenocarcinoma, high grade glioma, gastric carcinoma, acute promyelocytic leukemia [14], hepatocellular carcinoma [15] and intrahepatic cholangiocarcinoma [16]. ANXA2 also has been reported to be increased in colorectal cancer. Jin et al [17] used cDNA microarray gene expression profiling to characterize the transcriptional response to exposure of cultured mouse cerebral cortical neurons to hypoxia for 24h. The most strongly induced genes included ANXA2. All these certify that ANXA2 may play a certain role in tumor hypoxia adaptation.

Huang et al [13] detected the function of ANXA2 by siRNA to block the expression of ANXA2, showed a decrease in proliferation compared to the control cells and untransfected cells, and proved that ANXA2 is involved in apoptosis induced by p53. In addition, as a novel RNA-binding protein, ANXA2 may have an important physiological role in the regulation of c-myc mRNA. It binds directly to c-myc mRNA and up-regulates c-Myc protein [14]. There suggest that ANXA2 may also make effect on tumor cell proliferation, transformation and apoptosis.

In conclusion, our study combined a proteomic approach with RNAi technology to identify proteins associated with HIF1-α in SW480 cells in hypoxia. A number of proteins were found to be significantly altered in SW480siHIF1-α by comparing to SW480N cells. The changes are involved in energy metabolism, signal transduction and oxidative stress of cancer cells, which play a significant role in cell migration, hypoxia adaptation, cell proliferation and apoptosis. Where HIF1-α had significant association with PLD2 and ANXA2 in the twenty colon adenocarcinoma samples. This research prompts that there is a direct or indirect contact among them and the specific mechanism and function needs to be further studied in the future.

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