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Clinical importance of FASN in relation to HIF-1α and SREBP-1c in gastric adenocarcinoma

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ABSTRACT

Aims: Identifying alterations in lipid metabolism along gastric adenocarcinoma (GA) tumorigenesis pathways could lead to a new approach for potential diagnosis, efficient prediction and promising therapeutic strategies. This study aimed to identify the possible effect of HIF-1α on FASN and SREBP-1c regulation in GA.

Main methods: AGS cell line was cultured in normoxic and hypoxic conditions, and HIF-1α, FASN and SREBP-1c gene expression were analyzed by qRT-PCR and Western blot. Serum HIF-1α, FASN and insulin concentration were measured in 112 GA patients and 156 control cases by ELISA, and immunohistochemical method was employed to analyze SREBP-1c expression. Tissue mRNA expression of SREBP-1c, FASN and HIF-1α were determined by qRT-PCR.

Key findings: In vitro findings indicate upregulation of HIF-1α, FASN and SREBP-1c gene and protein expression in the hypoxic culture of AGS cells. High circulating levels of HIF-1α and FASN were significantly observed in GA patients compared to the controls. HIF-1α, SREBP-1c and FASN gene expression were higher in GA vs. controls. In addition, SREBP-1c protein level was enhanced in GA tissues compared to controls. Furthermore, elevated serum levels of HIF-1α and FASN and expression of HIF-1α, SREBP-1c and FASN genes were associated with unfavorable clinicopathological features such as diffuse type tumor and poor survival.

Significance: The results by correlating increased levels of FASN to those of HIF-1α and SREBP-1c are consistent with a possible up-regulation of FASN upon induction of HIF-1α through SREBP-1c.

1. Introduction

Gastric adenocarcinoma (GA) is the fifth most commonly diagnosed malignancy and the third most common cause of death due to cancer worldwide. It is a highly aggressive cancer and 5-year survival rate (5YSR) is usually < 30% [1]. In fact, the majority of these patients are identified in the late stages based on the endoscopic investigations and most widely used morphological classification introduced by Lauren [2], that was grouped GA as intestinal (well differentiated and slowly growing) and diffuse (poorly differentiated and aggressive) types. Accordingly, finding more sensitive and specific novel biomarkers for GA is required to assist in earlier diagnosis, classifying patients for personalized medicine and for improving the low survival outcome [3,4].

During cancer progression, tumor cells acquire comprehensive metabolic reprogramming, and tissue hypoxia is a prominent feature of solid tumors leading to cell metabolism adaptive changes. Hypoxia-inducible factor-1α (HIF-1α) is a key oxygen-regulated transcriptional activator, playing a fundamental role in the adaptation of tumor cells to hypoxia by upregulating the transcription of target genes related to multiple biological processes, including cell survival, proliferation, angiogenesis and anti-apoptosis [5–9]. In response to hypoxia, most malignant cells often exhibit a metabolic alteration toward anabolic pathways to synthesize their requirements that support the growth of

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cancer cells. Thus, abnormal fatty acid synthesis might be one of the critical biosynthetic tumor features that interferes with hypoxia and HIF [10]. As a sole mammalian enzyme capable of de novo lipogenesis (DNL), fatty acid synthase (FASN) was observed to be overexpressed in tumor tissues such as pancreas and breast cancers regardless of the extracellular lipid availability [11–13]. On the other hand, sterol regulatory element-binding protein (SREBP) is the central transcription factor to regulate fatty acid synthesis, suggesting that SREBP may have a pivotal role in regulation of lipogenesis by HIF-1α [14–16].

Based on this evidence, here we have examined the possible effect of HIF-1α expression on SREBP-1c and FASN regulation in GA tissues compared to control non-tumoral subjects as well as human gastric adenocarcinoma AGS cell line. There is no similar report involving HIF-1α, FASN and SREBP-1c in human GA, at least to the best of our knowledge. Moreover, in order to access a less invasive diagnostic approach, the role of circulating HIF-1α and FASN levels in GA were investigated. Lastly, the association between HIF-1α, SREBP-1c, FASN and GA clinicopathological features and survival rate was assessed.

2. Materials and methods

2.1. Human AGS cell culture and hypoxia induction

Human gastric adenocarcinoma cell line AGS was purchased from Pasteur Institute (Iran, Tehran) and cultured in RPMI-1640 supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum (FBS), at 37 °C and 5% CO_2 humidified incubator. To generate hypoxic condition, AGS cells were incubated in a chamber sustained at 1% O_2, 5% CO_2, and balanced with N_2 at 37 °C. AGS cells were cultured 24 h for mRNA expression analysis and 48 h for protein expression.

2.2. Western blot analysis

AGS cells in hypoxic condition were harvested and lysed with RIPA buffer and total protein content measured by Bradford protein assay. Forty microgram of extracted protein was separated using sodium dodecyl sulfate polyacrylamide 4%–12% gradient gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membranes (Roche). Blocking of non-specific bindings was done with skim milk solution in Tris-buffered saline Tween (TBST; 137 mM NaCl, 20 mM Tris/HCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. Then, we used primary antibodies against HIF-1α (1:1000 dilution, GeneTex, USA), FASN (1:800 dilution, GeneTex, USA), SREBP-1c (1:800 dilution, GeneTex, USA) and β-actin (1:500 dilution, GeneTex, USA) in 5% skim milk in TBST overnight at 4 °C incubation for membranes. Subsequently, horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody IgG (1:2000 dilution, GeneTex, USA) was used for treatment of blots for 1 h at room temperature. The bands in the blots were pictured by enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK). Image analysis software (ImageJ v1.51a) was employed for quantification of protein expression with normalized β-actin levels as an internal control for equal loading.

2.3. Patients information

Gastrointestinal tract endoscopy was performed for whom that presented at least one alarming dyspeptic complaint. A total of 112 patients with GA who were newly-diagnosed, previously untreated and histologically-confirmed GA by the endoscopy and pathology departments of Tabriz Medical University Hospitals between July 2015 and Aug 2016 were enrolled in this study. An additional 156 individuals were randomly selected as the non-tumoral control group from the endoscopy negative dyspeptic cases that were proven to have no identifiable endoscopic and histological evidence of peptic ulceration or gastric tumor. All patients and non-tumoral control group were naive to surgical resection, chemotherapy and radiotherapy. Subjects with concomitant conditions such as hyperlipidemia, thyroid and liver diseases, which can affect lipid metabolism, were also excluded. Samples were collected with approval from the Human Research Ethics Committees of Tarbiat Modares University (Tehran, Iran) (Ethic code: IR.TMU.REC.1394.184) and Liver and Gastrointestinal Diseases Research Center (Tabriz, Iran) (Ethic code: TBZMED.REC.1394.1074) for Clinical Investigation. All subjects, including potential controls, provided signed informed consent prior to enrollment in accordance with the declaration of Helsinki. Participants were followed up after diagnosis and completed a face-to-face questionnaire regarding socio-demographic features, clinical data and lifestyle factors.

A fasting venous blood sample was obtained prior to endoscopy; centrifuged at 3000 rpm for 5 min. Serum levels of glucose was measured using a standard method and leftover sera were stored at −80 °C. Gastric mucosa biopsies were also removed and an excision from each of the cases was stored immediately at −80 °C for RNA extraction. The remaining biopsies were fixed directly in 4% paraformaldehyde solution, paraffin-embedded and stained with Hematoxylin and Eosin (H& E). Subsequently, all histological sections were evaluated independently by two experienced gastrointestinal pathologists. According to the morphological classification introduced by Lauren [2], GA patients in this study are classified as intestinal (differentiated) and diffuse (poorly differentiated) major types. Histological grade and tumor site were determined as well.

2.4. ELISA

Circulating levels of HIF-1α and FASN were measured via enzyme-linked immunosorbent assay (ELISA) using the human HIF-1α (Thermo Scientific, USA) and FASN (Cloud Clone Crop, USA) ELISA kits, respectively, according to the manufacturer's instructions. Serum insulin was measured by a commercially available Insulin Human ELISA kit (Abcam, USA).

In summary, standards and serum samples were pipetted and incubated in the human HIF-1α, FASN and insulin 96-well capture plates. After washing, biotinylated anti-Human HIF-1α, FASN or insulin antibody was added and followed by incubation with HRP-conjugated streptavidin. The wells were again washed to remove all unbound enzymes, and color was generated by pouring tetramethyl-benzidine (TMB). Reactions were ended with stop solution (2 N sulfuric acid), and the density of the color was read at 450 nm. HIF-1α, FASN and insulin serum concentration were calculated using standard curves and all samples were analyzed in duplicate.

2.5. Immunohistochemistry

Slides used for immunohistochemistry (IHC) staining were generated from the original formalin-fixed, paraffin blocks that were made for the H&E staining. Paraffin-embedded tissues were cut into 5-μm-thick sections, then deparaffinized in xylene and rehydrated in a graded ethanol series. For epitope retrieval, sections were immersed in 10 mmol/l citrate buffer (pH 6.0) at 95 °C for 45 min, followed by blocking procedures according to the manufacturer's protocol. Incubation with the rabbit anti-human SREBP-1c antibody (diluted 1:500, Abcam, USA) was performed overnight at 4 °C. Subsequently, slides were washed with phosphate-buffered saline (PBS) and secondary antibody, (biotinylated anti-rabbit IgG) (R & D systems, Cell and Tissue Staining Kit, USA) was used. Detection was based on the formation of the avidin-biotin complex (ABC) with a primary antibody that reacts with gastric tissue SREBP-1c antigen. Therefore, the sections were treated with high sensitivity streptavidin conjugated to horseradish peroxidase (HSS-HRP) before visualization by diaminobenzidine (DAB) as the chromogen. After extensive washing, the counterstaining experiment was performed with Gill's hematoxylin solution (Sigma-Aldrich, HARLECO, USA) and eventually, the sections were dehydrated

and mounted.

The obtained IHC staining images were quantitatively analyzed using image analysis software (ImageJ v1.51a). At least 5 fields were processed from each section to verify the mean optical density (MOD) [17].

2.6. Real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze gene expression. Total RNA was isolated from the gastric tissues using the RNeasy Mini Kit (QIAGEN, Germany), and RNA concentration was determined by a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription (RT) was performed and complementary DNA (cDNA) synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany). The qRT-PCR experiments were done using PCR Mastermix (12.5 μl; Ampliqon, Denmark), cDNA templates (2 μl) (20 ng) and 1 μl each of the primers (0.2 μM). The volume of PCR mixture was set to 20 μl using the RNase-free water. The amplification reactions were performed using the ABI system (StepOnePlus™, ABI, USA) and all samples were done in triplicate. The following sequences of the primers were used: HIF-1α (Forward: 5′-TTCACCTGAGCCTAATAGTCC-3′, Reverse: 5′-CAAGTCTA AATCTGTGTCCTG-3′), FASN (Forward: 5′-CCATCTACAACATCGACA CCA-3′, Reverse: 5′-CTTCCACACTATGCTCAGGTAG-3′), SREBP-1c (Forward: 5′-CTGGTCTACCATAAGCTGCAC-3′, Reverse: 5′-GACTGGT CTTCACTCTCAATG-3′) and β-actin (Forward: 5′-TGAGACCTTCAACA CCCCAGCCATG-3′, Reverse: 5′-CGTAGATGGGCACAGTGTGGGTG-3′).

The PCR cycling conditions composed of an initial denaturation step at 95 °C for 15 min followed by 40 cycles of: 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The β-actin gene amplification was used as an internal control and expression of HIF-1α, SREBP-1c and FASN genes was calculated as fold change.

2.7. Survival analysis

Overall survival curves were plotted by the Kaplan-Meier method to determine the time of diagnosis to the date of death, and comparisons were evaluated by the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model on all significant characteristics measured for univariate analysis.

2.8. Statistical analysis

Statistical tests were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA) and SPSS version 22 (SPSS, Inc., Chicago, IL, USA). Differences between experimental groups were evaluated using unpaired Student's *t*-tests and Mann-Whitney *U* test, where appropriate. Frequency variations were assessed by Chi-square test. The Pearson and Spearman correlation tests were applied to analyze the association between categorical variables. Additionally, the receiver operator characteristic (ROC) curve was constructed to estimate diagnostic sensitivity and specificity values. All data were presented as mean ± SD and a cut-off value of $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Induction of HIF-1α, FASN and SREBP-1c genes through hypoxia in the human gastric adenocarcinoma AGS cell line

To investigate the hypoxia effects on the expression of HIF-1α, FASN and SREBP-1c genes, we cultured the human gastric adenocarcinoma AGS cell line under hypoxic or normoxic conditions. As shown in Fig. 1, our results indicate the upregulation of HIF-1α (6.2 fold), FASN (8.8 fold) and SREBP-1c (4.6 fold) genes in hypoxia compared to normoxia (Fig. 1A). Similarly, protein expression of HIF-1α (4.4 fold), FASN (6.0 fold) and SREBP-1c (3.6 fold) were increased in the AGS cell line under hypoxic condition (Fig. 1B and C). These findings suggest that the expression of FASN and SREBP-1c genes are positively correlated with hypoxia and HIF-1α upregulation.

3.2. Clinical experiments

This study included 112 GA patients (36 women and 76 men), with ages ranging from 32 to 88 years old (median, 70 years) and 156 control cases (54 women and 102 men, age range 48–87 years, median, 65 years). The clinicopathological data are depicted in Table 1.

To investigate the mechanism by which HIF-1α upregulates the expression of FASN, the critical enzyme of fatty acid synthesis in the cytoplasm, serum levels of HIF-1α, FASN and insulin concentrations were compared between GA patients and non-tumoral control subjects (Fig. 2). Circulating levels of HIF-1α and FASN were significantly higher in subjects with GA (HIF-1α: 148.8 ± 58.4 pg/ml and FASN: 16.3 ± 10.5 ng/ml) compared to control group (HIF-1α: 101.9 ± 82.9 pg/ml and FASN: 11.2 ± 7.4 ng/ml) ($p = 0.001$ and $p = 0.03$), respectively. However, there was no statistically significant difference in glucose ($p = 0.42$) and insulin ($p = 0.1$) serum levels between the two groups.

Immunohistochemical analysis was employed to analyze SREBP-1c expression in GA tumors and control gastric biopsies. SREBP-1c positive immunostaining was detected in both cytoplasm and nucleus of the cells. Photomicrographs of SREBP-1c staining in the different gastric tissues are shown in Fig. 3A–D. SREBP-1c levels were significantly increased in GA tissues (0.16 ± 0.07) compared to controls (0.09 ± 0.06, $p = 0.003$) according to analysis of the staining density (Fig. 3E).

To determine the expression of FASN and SREBP-1c, two target genes of HIF-1α activation, FASN, SREBP-1c and HIF-1α mRNA levels were measured in tissues by qRT-PCR. Therefore, the results obtained from ELISA and immunohistochemistry were further confirmed by qRT-PCR. The relative mRNA expression of HIF-1α (fold change: 12.69), SREBP-1c (fold change: 6.77) and FASN (fold change: 20.91) genes were higher in GA tissues vs. control group (Fig. 4).

Serum level of FASN, and SREBP-1c protein expression in GA tissues were correlated with HIF-1α augmentation in GA patients ($p = 0.0001$ and $p = 0.0001$, respectively), as shown in Fig. 5. Taken together, our findings support that HIF-1α could stimulate FASN upregulation along with SREBP-1c activation in GA, and simultaneous measurement of serum HIF-1α and FASN could be used as practical targets for the noninvasive early detection of GA.

In order to determine the clinical role of fatty acid anabolic pathway activation in the progression of GA, the relationship between HIF-1α, SREBP-1c and FASN levels were assessed and matched with clinicopathological attributes (Figs. 2–5). Using the Lauren classification, FASN circulating levels were elevated in cases with diffuse (25.8 ± 13.5 ng/ml) compared to intestinal type of GA (13.1 ± 7.39 ng/ml) ($p = 0.004$). Therefore, FASN levels should be related with adverse histopathological findings in subjects with GA (Fig. 2D). In addition, there was a significant difference between diffuse (192.3 ± 50.5 pg/ml) and intestinal (136.8 ± 54.4 pg/ml) subgroups concerning HIF-1α status in serum ($p = 0.026$) (Fig. 2C). Our IHC findings show that SREBP-1c protein is not notably upregulated in diffuse type (0.19 ± 0.12) of GA compared to intestinal type (0.15 ± 0.05) ($p = 0.27$) (Fig. 3F), although SREBP-1c protein expression is significantly upregulated in GA patients. Additionally, the majority of intestinal-type of GA specimens showed lower mRNA expressions of HIF-1α (fold change: 10.12), FASN (fold change: 17.54) and SREBP-1c (fold change: 5.36) when compared to diffuse type malignancy (fold change: 15.25, fold change: 27.14, fold change: 8.34, respectively) ($p = 0.011$, 0.014 and 0.003, respectively) (Fig. 4B–D). These findings suggest that the expression of HIF-1α, SREBP-1c and FASN are gradually upregulated while GA progresses to the worse

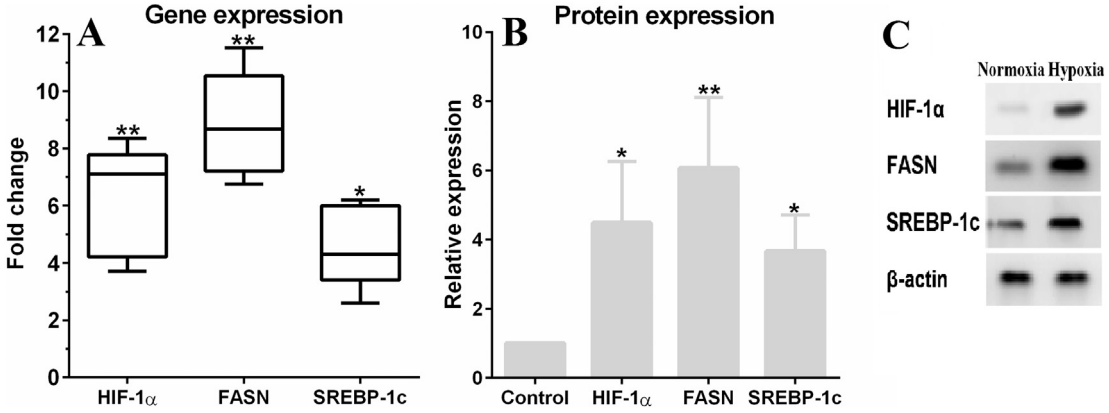

Fig. 1. Hypoxia induces the expression of HIF-1α, FASN and SREBP-1c genes in the human gastric adenocarcinoma AGS cell line. The human gastric adenocarcinoma AGS cell line was cultured in 24-well plates under normoxic or hypoxic condition for 24 and 48 h. HIF-1α, FASN and SREBP-1c genes were analyzed by qRT-PCR (A) and Western blot in harvested cells (B and C). Our results showed that gene and protein expression of HIF-1α was significantly upregulated in hypoxia compared to normoxia. Moreover, both gene and protein expression of FASN and SREBP-1c were significantly increased in hypoxic condition. Protein expression was quantified using ImageJ software and corrected for the levels of β-actin in the same samples. The mean value in normoxic condition AGS cells was set at 1 and other results were standardized to these values (fold change). Data are presented as mean ± SD of at least three independent experiments. * and ** indicates, p values < 0.05 and 0.01 respectively. HIF-1α (hypoxia inducible factor-1α), FASN (fatty acid synthase), SREBP-1c (sterol regulatory element-binding protein).

Table 1
Clinicopathological features of GA patients and control subjects.

Parameters	GA patients	Control subjects	p-Value
Sex	n = 112	n = 156	
Female	36 (32%)	54 (34.6%)	
Male	76 (68%)	102 (65.4%)	0.67[a]
Age	Median: 70 (32–88)	Median: 65 (48–87)	0.63[b]
BMI	22.06 ± 2.79	26.32 ± 3.96	0.000[b]
Lauren classification			
Intestinal	80 (71%)	–	
Diffuse	28 (25%)	–	
Histological grade			
G1	24 (21.4%)	–	
G2	56 (50%)	–	
G3	24 (21.4%)	–	
G4	4 (3.6%)	–	
Glucose	5.08 ± 1.61 mm/l	5.37 ± 1.39 mm/l	0.42[b]
Insulin	6.09 ± 1.54 μIU/ml	5.64 ± 0.68 μIU/ml	0.1[b]

[a] Chi-square test.
[b] *t*-Test, GA (Gastric Adenocarcinoma), BMI (Body Mass Index), G (Grade).

clinical phase.

Survival analyses using the Kaplan-Meier method for the 2-years follow-up was performed according to clinicopathological variables and serum levels of HIF-1α and FASN, as well as SREBP-1c protein expression. In univariate analysis, circulating FASN and HIF-1α serum levels and SREBP-1c protein expression have significant predictive values for overall survival, together with Lauren classification (p = 0.0001, 0.0001, 0.0001 and 0.0001, respectively) (Fig. 6). The following factors were not significantly associated with prognosis: age, sex, and tumor location (p = 0.07, 0.32 and 0.12). Furthermore, multivariate analysis indicated that increased serum levels of FASN (p = 0.034, CI = 1.035–2.358), HIF-1α (p = 0.008, CI = 1.201–3.463), and SREBP-1c protein expression (p = 0.006, CI = 1.201–2.914) are associated with poor prognosis, suggesting that these molecules could serve as prognostic factors for overall survival in GA.

In addition, receiver operating characteristic (ROC) analysis was performed to describe the individual diagnostic potential of serum levels of HIF-1α and FASN as measured by ELISA. The values represented as the area under the curve (AUC) of HIF-1α and FASN were 0.725 (95% CI: 0.604–0.845, p < 0.002) and 0.651 (95% CI: 0.515–0.787, p < 0.037), respectively.

4. Discussion

GA is among the most common lethal malignancies worldwide [1,3,4]. The revelation of lipid metabolic alterations along GA tumorigenesis pathways could lead to a new approach for earlier detection, efficient prediction and favorable therapeutic strategies.

Deregulated growth is the most apparent feature of malignant cells resulting in the increased expenses of oxygen and induction of hypoxia in solid tumors. This induces the stabilization and activation of the HIF-1α, a crucial transcriptional regulator in the adaptation of tumor cells to pathologic hypoxia [6,7]. Our study results not only further depict that HIF-1α gene expression is significantly enhanced in patients with GA, but also found that elevated level of circulating HIF-1α was indeed correlated with GA, considering clinicopathological parameters such as undifferentiated tumor and poor patient survival. This report is consistent with previous data in other cancer types proposing that HIF-1α overexpression occurs early in tumorigenesis [18]. Moreover, it has been shown that in some cancers, HIF-1α upregulation associated directly with loss of differentiation, highly aggressive disease and poor patient outcome [5,19]. Here, we also demonstrated that local gene expression of FASN is increased in patients with GA. In support of this idea, former in vitro studies showed that FASN upregulation could be a substantial factor in cellular growth, transformation and carcinomatous potential, and could thus be considered to act as an oncogene-like contributor [11,13]. A previous research showed that FASN interacts with caveolin-1 on the membrane microdomains of prostate cancer cells [20]. It was observed that proliferative structures like neuronal stem cells require FASN to guarantee their rapid growth [21,22]. Our data suggested that low oxygen availability in GA tumor tissues along with HIF-1α induction might lead to upregulation of FASN. Furthermore, despite being an intracellular protein, we found circulating FASN level in GA cases was significantly higher compared to controls, suggesting that the gastric tumor was the primary source of systematic FASN upregulation in our GA patients. Based on other works, elevated FASN levels have been identified in the sera of pancreas and breast cancers [12,13]; that is in line with our findings. We also observed a significantly high expression of FASN in subjects with diffuse rather than intestinal type of GA. Recent studies indicated that FASN is a prerequisite for rapid cell growth, higher proliferation rates and tumor survival, forming an aberrant and vicious proliferation cycle that is involved in cell phenotype transformation and tumor development [23–25]. Moreover, our data demonstrated that the elevated blood level of FASN in GA patients correlates with higher undifferentiation, shorter

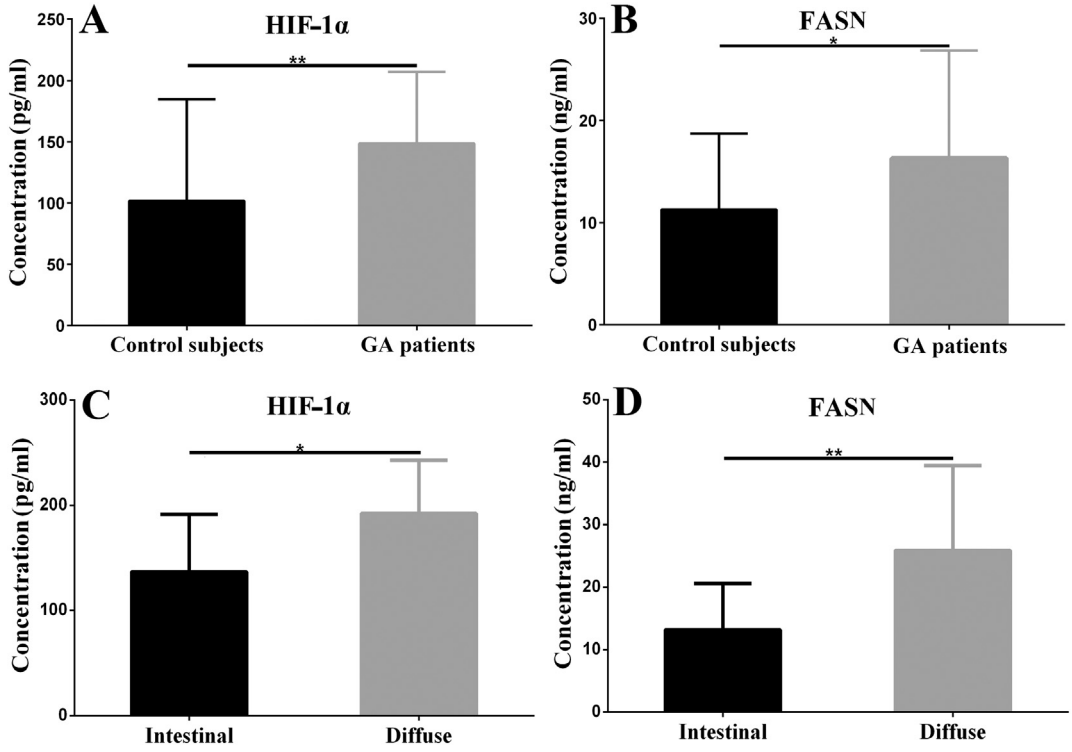

Fig. 2. Serum HIF-1α and FASN levels increased in GA patients. The concentration of serum HIF-1α and FASN in GA patients and controls were measured by ELISA method. The data demonstrated that HIF-1α and FASN levels were significantly higher in GA patients than in control subjects (A and B). Statistical analysis showed significant upregulation of HIF-1α and FASN concentration in diffuse type of GA patients compared to intestinal type (C and D). The data are presented here as (Mean ± SD). * and ** means, p values < 0.05 and 0.01, respectively. GA (gastric adenocarcinoma), HIF-1α (hypoxia inducible factor-1α), FASN (fatty acid synthase).

time to death and poor prognosis that is in parallel with other works [11,12]. Altogether, we suggested that FASN overexpression is intensely related to the development of GA.

In the present study, fasting blood glucose and insulin levels were not significantly different between GA patients and control cases. Normal mammalian cells typically acquire fatty acids from the circulation owing to insulin, which is the most lipogenic hormone in the subset of adult tissues, including liver, adipose and lactating mammary

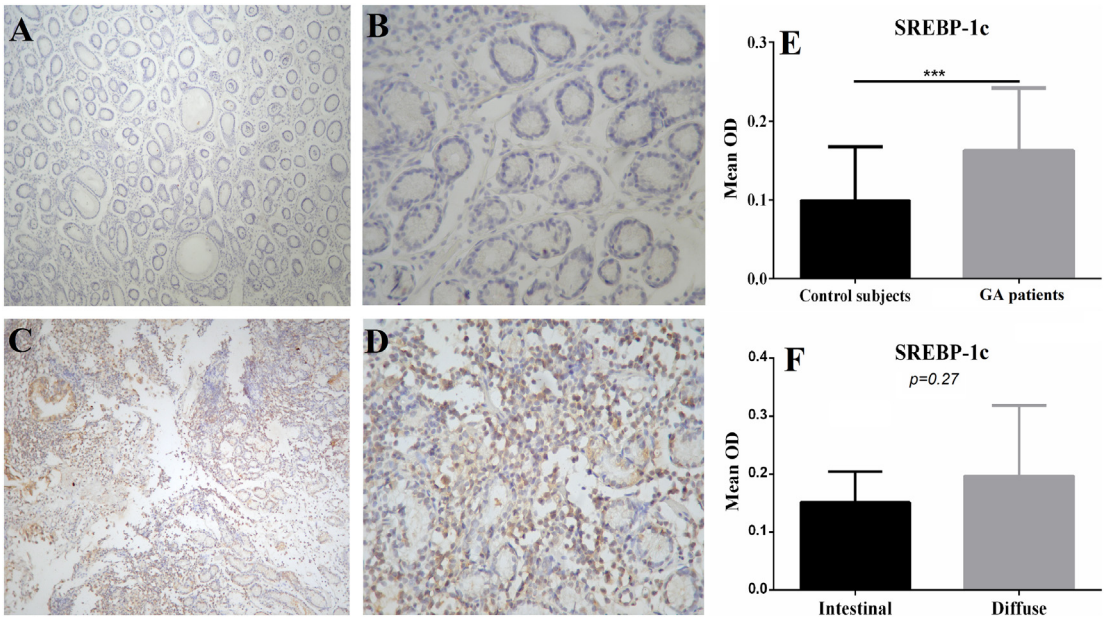

Fig. 3. Increased expression of SREBP-1c protein in GA tissues. SREBP-1c expression in GA and control subject tissues was determined by IHC method. Non-tumoral subject (A, 40× and B, 400×) showed a lower staining of SREBP-1c, but GA (C, 40× and D, 400×) showed higher density staining. Our results showed a significant upregulation of SREBP-1c protein expression in GA patients (E). In addition, we did not observe a significant difference in SREBP-1c expression between intestinal and diffuse types of GA (F). The data are presented here as (Mean ± SD). *** means, p value < 0.001. GA (gastric adenocarcinoma), SREBP-1c (sterol regulatory element-binding protein), MOD (mean optical density).

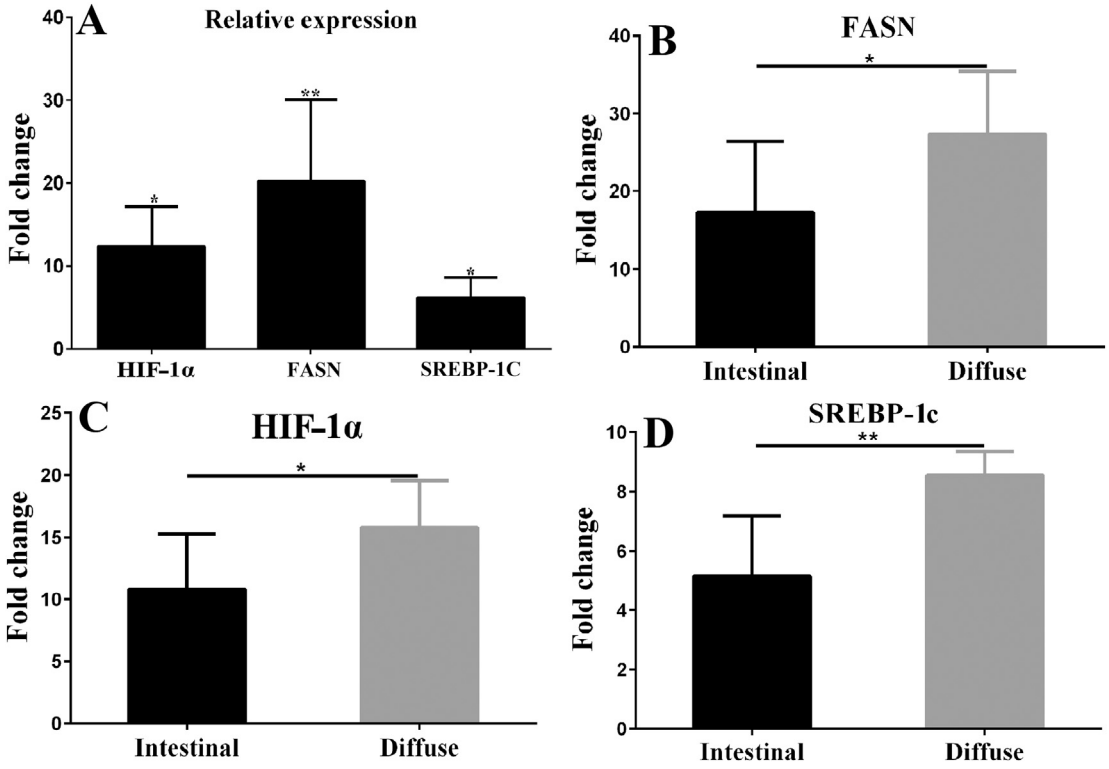

Fig. 4. Gene expression of FASN and SREBP-1c were increased following activation of HIF-1α gene in GA. The mRNA was extracted from GA patient and control subject tissues and the relative expression of HIF-1α, FASN and SREBP-1c genes were measured using qRT- PCR. The relative expression of these genes was significantly increased in GA tissues compared to controls (A). In addition, our data showed significant upregulation of these genes expression in diffuse vs. intestinal type of GA (B, C and D). The β-actin gene amplification was used as an internal control. The data are presented here as (Mean ± SD) of fold changes. All gene expression assays were done in triplicate. * and ** means, p values < 0.05 and 0.01, respectively. HIF-1α (hypoxia inducible factor-1α), FASN (fatty acid synthase), SREBP-1c (sterol regulatory element-binding protein).

gland [11,24]. In contrast, it was suggested that some cancer types depend on de novo fatty acid biosynthesis that could be regulated by the transcription factor SREBP-1c [26]. As a result, unrestricted fatty acid anabolism could be observed in cancers [11,14,15], unaffected by the extracellular lipid availability and regulatory hormonal motivation [24]. Previous studies found that SREBP acts as an oxygen sensor in fission yeast [27] and to be required for the conservation of cell size in *Drosophila. Melanogaster* [28]. SREBP-1c is a transcription factor of the basic helix-loop-helix leucine zipper (bHLHLZ) that indeed leads to lipid homeostasis in mammals. It has a regulatory role during mitosis, showing that the expression of lipogenic genes is a prerequisite for cell-cycle progression. To address the pathway underlying HIF-1α-induced FASN upregulation, we tested SREBP-1c expression in GA. Based on our current findings, SREBP-1c gene upregulated in GA tumor tissues compared to controls. Elevated mRNA expression of SREBP-1c was also supported by our IHC data, which revealed SREBP-1c overexpression in the majority of tumor sections. Moreover, the SREBP-1c expression was associated significantly with patient shorter survival. In keeping with our findings, SREBP-1c has been shown to be present in atypically hyperplastic tissues including glioblastoma multiforme and pancreatic cancers [14,15]. In vitro studies demonstrate that SREBP-1c encourages cell growth and metastasis [28–30]. In the present study, we also showed that the HIF-1α overexpression was accompanied with the highly expressed genes of FASN and SREBP-1c, indicating that SREBP-

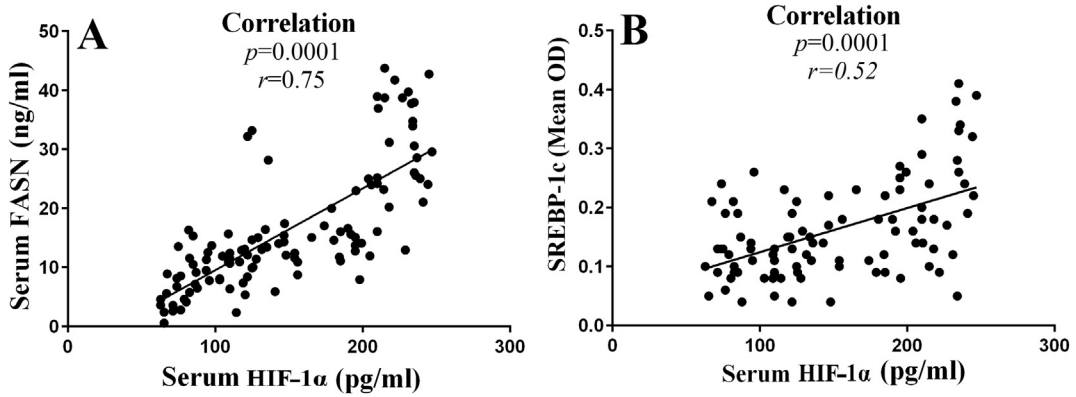

Fig. 5. Serum FASN and protein expression of SREBP-1c correlated with serum HIF-1α. Our findings showed that upregulation of serum HIF-1α in GA patients was significantly associated with serum FASN (A) and protein expression of SREBP-1c (B). HIF-1α (hypoxia inducible factor-1α), FASN (fatty acid synthase), SREBP-1c (sterol regulatory element-binding protein), MOD (mean optical density).

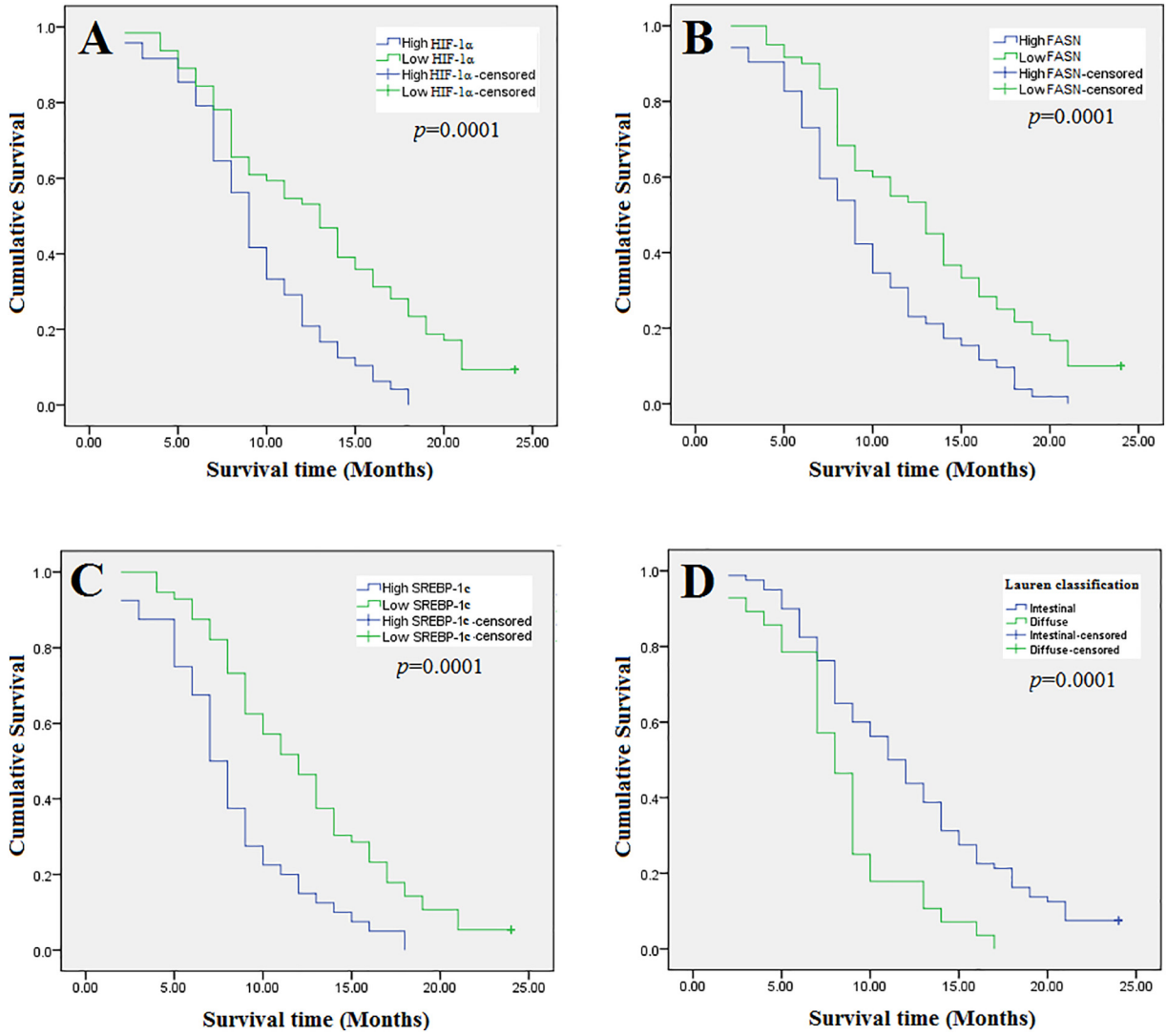

Fig. 6. Kaplan-Meier 2-years univariate analysis of overall survival. Cumulative survival of GA patients with reference to serum levels of HIF-1α (A), FASN (B) and SREBP-1c protein expression (C) as well as Lauren classification (D). Differences between the two groups were evaluated with the Kaplan-Meier curves. Log-rank p values are given. HIF-1α (hypoxia inducible factor-1α), FASN (fatty acid synthase), SREBP-1c (sterol regulatory element-binding protein).

1c and FASN genes could be subject to the same regulatory mechanisms in the human GA progression. In addition, our in vitro studies provide evidence that hypoxia induced-HIF-1α could be responsible for the stimulation of FASN, and SREBP-1c may have a direct role in hypoxia-induced FASN expression in human gastric adenocarcinoma AGS cell line. To confirm our findings, it was observed that SREBP-1c could be as a target gene of HIF-1α [15,16]. Furthermore, silencing of SREBP-1c expression dramatically decreases lipogenesis and inducing apoptosis in some cancers types [31,32]. Consequently, HIF-1α induction accompanied with FASN and SREBP-1c upregulation seems to be a survival approach of GA cells in hypoxic condition, by metabolic alteration toward lipogenic pathway.

5. Conclusion

In conclusion, overexpression of HIF-1α was consistent with increased FASN and SREBP-1c gene and protein expression in GA patients and human gastric adenocarcinoma AGS cell line in hypoxic condition. Therefore, our study support that HIF-1α could stimulate FASN upregulation accompanied with SREBP-1c activation in GA. It was demonstrated that the extracellular levels of HIF-1α and FASN, and SREBP-1c protein expression may have diagnostic and prognostic values in GA patients, together with Lauren classification. Further studies are required to validate the utility of HIF-1α, SREBP-1c and FASN as early bona fide diagnosis and prediction of GA.

Conflicts of interest

The authors declare no financial competing interests.

Ethical statement and informed consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent to be included in the study, or the equivalent, was obtained from all patients.

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References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (6) (2018) 394–424.

[2] P. Lauren, The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification, Acta Pathol. Microbiol. Scand. 64 (1) (1965) 31–49.

[3] S.M. Mousavi, M.M. Gouya, R. Ramazani, M. Davanlou, N. Hajsadeghi, Z. Seddighi, Cancer incidence and mortality in Iran, Ann. Oncol. 20 (3) (2008) 556–563.

[4] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (5) (2015) 359–386.

[5] P. Birner, M. Schindl, A. Obermair, C. Plank, G. Breitenecker, G. Oberhuber, Overexpression of hypoxia-inducible factor 1α is a marker for an unfavorable prognosis in early-stage invasive cervical cancer, Cancer Res. 60 (17) (2000) 4693–4696.

[6] B. Keith, R.S. Johnson, M.C. Simon, HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression, Nat. Rev. Cancer 12 (1) (2012) 9.

[7] G.L. Semenza, Hypoxia-inducible factors in physiology and medicine, Cell 148 (3) (2012) 399–408.

[8] Y. Jia, R. Hu, P. Li, Y. Zheng, Y. Wang, X. Ma, DEC1 is required for anti-apoptotic activity of gastric cancer cells under hypoxia by promoting Survivin expression, Gastric Cancer 21 (4) (2018) 632–642.

[9] H. Kim, Q. Lin, Z. Yun, The hypoxic tumor microenvironment in vivo selects the tumor cells with increased survival against genotoxic stresses, Cancer Lett. 431 (2018) 142–149.

[10] J.A. Menendez, L. Vellon, B.P. Oza, R. Lupu, Does endogenous fatty acid metabolism allow cancer cells to sense hypoxia and mediate hypoxic vasodilatation? Characterization of a novel molecular connection between fatty acid synthase (FAS) and hypoxia-inducible factor-1α (HIF-1α)-related expression of vascular endothelial growth factor (VEGF) in cancer cells overexpressing her-2/neu oncogene, J. Cell. Biochem. 94 (5) (2005) 857–863.

[11] D. Buckley, G. Duke, T.S. Heuer, M. O'farrell, A.S. Wagman, W. McCulloch, G. Kemble, Fatty acid synthase–modern tumor cell biology insights into a classical oncology target, Pharmacol. Ther. 177 (2017) 23–31.

[12] K. Walter, S.-M. Hong, S. Nyhan, M. Canto, N. Fedarko, A. Klein, M. Griffith, N. Omura, S. Medghalchi, F. Kuhajda, Serum fatty acid synthase as a marker of pancreatic neoplasia, Cancer Epidemiol. Biomark. Prev. (2009) 1055–9965 (EPI-1009-0144).

[13] A. Vazquez-Martin, R. Colomer, J. Brunet, R. Lupu, J. Menendez, Overexpression of fatty acid synthase gene activates HER1/HER2 tyrosine kinase receptors in human breast epithelial cells, Cell Prolif. 41 (1) (2008) 59–85.

[14] Y. Sun, W. He, M. Luo, Y. Zhou, G. Chang, W. Ren, K. Wu, X. Li, J. Shen, X. Zhao, SREBP1 regulates tumorigenesis and prognosis of pancreatic cancer through targeting lipid metabolism, Tumor Biol. 36 (6) (2015) 4133–4141.

[15] C. Lewis, C. Brault, B. Peck, K. Bensaad, B. Griffiths, R. Mitter, P. Chakravarty, P. East, B. Dankworth, D. Alibhai, SREBP maintains lipid biosynthesis and viability of cancer cells under lipid-and oxygen-deprived conditions and defines a gene signature associated with poor survival in glioblastoma multiforme, Oncogene 34 (40) (2015) 5128.

[16] E. Furuta, S.K. Pai, R. Zhan, S. Bandyopadhyay, M. Watabe, Y.-Y. Mo, S. Hirota, S. Hosobe, T. Tsukada, K. Miura, Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1, Cancer Res. 68 (4) (2008) 1003–1011.

[17] D. Huang, T. Li, X. Li, L. Zhang, L. Sun, X. He, X. Zhong, D. Jia, L. Song, G.L. Semenza, HIF-1-mediated suppression of acyl-CoA dehydrogenases and fatty acid oxidation is critical for cancer progression, Cell Rep. 8 (6) (2014) 1930–1942.

[18] L.P. Schwab, D.L. Peacock, D. Majumdar, J.F. Ingels, L.C. Jensen, K.D. Smith, R.C. Cushing, T.N. Seagroves, Hypoxia-inducible factor 1α promotes primary tumor growth and tumor-initiating cell activity in breast cancer, Breast Cancer Res. 14 (1) (2012) R6.

[19] J. He, Y. Hu, M. Hu, S. Zhang, B. Li, The relationship between the preoperative plasma level of HIF-1α and clinic pathological features, prognosis in non-small cell lung cancer, Sci. Rep. 6 (2016) 20586.

[20] D. Di Vizio, R.M. Adam, J. Kim, R. Kim, F. Sotgia, T. Williams, F. Demichelis, K.R. Solomon, M. Loda, M.A. Rubin, Caveolin-1 interacts with a lipid raft-associated population of fatty acid synthase, Cell Cycle 7 (14) (2008) 2257–2267.

[21] M. Knobloch, S.M. Braun, L. Zurkirchen, C. Von Schoultz, N. Zamboni, M.J. Araúzo-Bravo, W.J. Kovacs, Ö. Karalay, U. Suter, R.A. Machado, Metabolic control of adult neural stem cell activity by Fasn-dependent lipogenesis, Nature 493 (7431) (2013) 226.

[22] H.J. Lee, J.M. Ryu, Y.H. Jung, S.Y. Oh, S.J. Lee, H.J. Han, Novel pathway for hypoxia-induced proliferation and migration in human mesenchymal stem cells: involvement of HIF-1α, FASN, and mTORC1, Stem Cells 33 (7) (2015) 2182–2195.

[23] J. Li, J.-X. Cheng, Direct visualization of de novo lipogenesis in single living cells, Sci. Rep. 4 (2014) 6807.

[24] M. Huang, A. Koizumi, S. Narita, T. Inoue, N. Tsuchiya, H. Nakanishi, K. Numakura, H. Tsuruta, M. Saito, S. Satoh, Diet-induced alteration of fatty acid synthase in prostate cancer progression, Oncogenesis 5 (2) (2016) e195.

[25] A.M. Gonzalez-Guerrico, I. Espinoza, B. Schroeder, C.H. Park, K.C. Mohan, A. Khurana, B. Corominas-Faja, E. Cuyàs, T. Alarcón, C. Kleer, Suppression of endogenous lipogenesis induces reversion of the malignant phenotype and normalized differentiation in breast cancer, Oncotarget 7 (44) (2016) 71151.

[26] W. Shao, P.J. Espenshade, Expanding roles for SREBP in metabolism, Cell Metab. 16 (4) (2012) 414–419.

[27] A.L. Hughes, B.L. Todd, P.J. Espenshade, SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast, Cell 120 (6) (2005) 831–842.

[28] T. Porstmann, C.R. Santos, B. Griffiths, M. Cully, M. Wu, S. Leevers, J.R. Griffiths, Y.-L. Chung, A. Schulze, SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth, Cell Metab. 8 (3) (2008) 224–236.

[29] Z. Shi, Q. Zhou, S. Gao, W. Li, X. Li, Z. Liu, P. Jin, J. Jiang, Silibinin inhibits endometrial carcinoma via blocking pathways of STAT3 activation and SREBP1-mediated lipid accumulation, Life Sci. 217 (2019) 70–80.

[30] R. Bartz, L.P. Sun, B. Bisel, J.H. Wei, J. Seemann, Spatial separation of Golgi and ER during mitosis protects SREBP from unregulated activation, EMBO J. 27 (7) (2008) 948–955.

[31] A.A. Gholkar, K. Cheung, K.J. Williams, Y.-C. Lo, S.A. Hamideh, C. Nnebe, C. Khuu, S.J. Bensinger, J.Z. Torres, Fatostatin inhibits cancer cell proliferation by affecting mitotic microtubule spindle assembly and cell division, J. Biol. Chem. C116 (2016) 737346.

[32] N. Li, Z.S. Zhou, Y. Shen, J. Xu, H.H. Miao, Y. Xiong, F. Xu, B.L. Li, J. Luo, B.L. Song, Inhibition of the sterol regulatory element-binding protein pathway suppresses hepatocellular carcinoma by repressing inflammation in mice, Hepatology 65 (6) (2017) 1936–1947.