High Prevalence of Fatty Acid Synthase Expression in Colorectal Cancers in Middle Eastern Patients and Its Potential Role as a Therapeutic Target

Shahab Uddin, PhD1,4, Azhar R. Hussain, MBBS1,4, Maqbool Ahmed, PhD1, Jehad Abubaker, PhD1, Nasser Al-Sanea, MD2, Alaa AbdulJabbar, MD1, Luai H. Ashari, MD1, Samar Alhomoud, MD1, Fouad Al-Dayel, MD1, Prashant Bavi, MD1 and Khawla S. Al-Kuraya, MD, FCAP1

OBJECTIVES: Many human epithelial cancers, particularly those with a poor prognosis, express high levels of fatty acid synthase (FASN), a key metabolic enzyme linked to synthesis of membrane phospholipids in cancer cells. Overexpression of FASN is linked with activation of the phosphatidylinositol-3'-kinase (PI3 K)/AKT pathway. However, the role of FASN in colorectal cancer (CRC) has not been fully elucidated. We investigated the expression of FASN and determined its functional association with the PI3/AKT pathway in CRC.

METHODS: Expression of FASN and its associated targets were studied by immunohistochemistry on 448 CRC tumors in a tissue microarray (TMA) format. Analysis of apoptosis and cell cycle was evaluated in vitro using CRC cell lines by flow cytometry and DNA fragmentation assays. Protein expression was determined by immunohistochemistry and western blotting. In vivo xenograft studies were performed using CRC cell lines and NUDE mice.

RESULTS: Correlation of FASN with various clinicopathological parameters on 448 CRC samples was assessed. Activated AKT was found in 294/409 (71.9%) of CRC and was associated with FASN overexpression. FASN expression was observed in 27.1% (109/403) of Middle Eastern CRC. Additionally, FASN expression was significantly more common in tumors characterized by microsatellite instability (MSI) than in those characterized by microsatellite stability (MSS) (P<0.01). Our in vitro data using HCT-15, an MSI CRC cell line, showed a better apoptotic response after inhibition of FASN activity as compared with Colo-320, an MSS CRC cell line. Finally, treatment of HCT-15 cell line xenografts with C-75 resulted in growth inhibition of tumors in NUDE mice via downregulation of FASN and AKT activity.

CONCLUSIONS: These data identify FASN as a potential biomarker and a novel therapeutic target in distinct molecular subtypes of CRC.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

Am J Gastroenterol 2009; 104:1790–1801; doi: 10.1038/ajg.2009.230; published online 2 June 2009

INTRODUCTION
Colorectal cancer (CRC) still has a high morbidity and mortality. It is now established that the subtype of CRC characterized by microsatellite instability (MSI) follows a pathway different from that characterized by microsatellite stability (MSS) (1,2). The clinicopathological characteristics are becoming clear, but specific genetic and therapeutic differences remain to be fully defined. Significant improvements have been made in the management of this disease mainly through the introduction of adjuvant chemotherapy agents like fluorouracil and oxaliplatin (3). More recently, advances in understanding of tumor biology have led to the development of targeted therapies (4), allowing progress in the treatment of colorectal cancer (5).
One of the promising targets for therapeutic intervention is fatty acid synthase (FASN), which is a multifunctional enzyme that catalyses the terminal steps in the synthesis of long chain saturated fatty acid. In normal cells, energy balance is physiologically important in FASN regulation and high carbohydrate/low fat diets upregulate FASN (6).

A wide variety of tumors and their precursor lesions undergo exacerbated de novo biosynthesis of fatty acids irrespective of the level of circulating lipids. Neoplastic lipogenesis is reflected by significantly increased activity and coordinate expression of several lipogenic enzymes such as FASN that is upregulated in most solid tumors (7–11). FASN appears to provide a selective proliferative advantage as its overexpression showed to correlate with poor prognosis in breast and prostate cancers and is found elevated in the blood of cancer patients (12–14). Furthermore, inhibition of FASN activity is selectively cytotoxic to cancer cells in vitro and in vivo (15,16).

Upregulation of FASN expression in cancer cells has been linked to phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway (17–20). Activation of PI3-kinase pathway recruits a number of signaling proteins including protein kinase B (also known as AKT). On recruitment, AKT becomes phosphorylated/activated and exerts its anti-apoptotic activity through phosphorylation of downstream targets such as Bad, FOXO, and GSK3 (21,22).

Interestingly, PI3K/AKT has been shown to modulate the activity of SKP2, an ubiquitin ligase that degrade the p27kip1 by proteasomes (23). In addition, PI3K pathway has been shown to be capable of negatively regulating FASN-induced cell death (24,25). Therefore, we sought to assess activation of PI3K/AKT pathway in CRC, whether this activation correlates with increased FASN expression and relation to other clinicopathological parameters in a large cohort of Saudi CRC using tissue microarray (TMA) technology. We next examined the effect of C-75, a synthetic slow binding inhibitor of FASN activity, on CRC cell lines both in vivo and in vitro. All together, our findings strongly suggest that a tight functional association between FASN and AKT is taking place in a subset of CRC and that FASN expression can be a useful biomarker in this subset of CRC.

METHODS

Patient selection and TMA construction
In total, 448 patients with CRC diagnosed between 1990 and 2006 with available formalin fixed paraffin embedded tumor tissues were selected from King Faisal Specialist Hospital and Research Centre. All patients diagnosed with colorectal cancer during this period were randomly selected and only those patients were excluded where formalin fixed paraffin embedded tissues were not available. All samples were analyzed in TMA format. TMA construction was performed as described earlier (26). Three 0.6 mm cores of CRC were arrayed from each case. All clinical data and long-term follow-up was provided by colorectal unit, Department of Surgery, King Faisal Specialist Hospital and Research Centre. The institutional review board of King Faisal Specialist Hospital and Research Center approved the study.

Immunohistochemistry
TMA slides were processed and stained manually. The streptavidin-biotin peroxidase technique with diaminobenzidine as chromogen was applied. For antigen retrieval, Dako Target Retrieval Solution pH 9.0 was used. The list of primary antibodies used are listed in Supplementary Table 1. Immunohistochemistry scoring was done in a blinded manner and TMA spots were scored under a 20×0.70 objective on an Olympus BX 51 microscope (Olympus America Inc., Center Valley, PA).

FASN expression was categorized as negative (no or weak expression), moderate (1+), or strong (2+) as has been reported earlier (11). Adipose tissue served as internal positive control for FASN expression. p-AKT scoring was done as described earlier (27,28). Cases were considered positive if 50% or more of tumor cells were stained positive for p27kip1 and SKP2. This cutoff was chosen based on prior analysis on some of the markers and similar cutoff used by others (29).

Statistics
The software used for statistical analysis was statview 5.0 (SAS Institute Inc., NC). Survival curves were constructed by Kaplan–Meier method and multivariate analysis by Cox regression; P values <0.05 were considered significant. Two-sided tests were used throughout all the analyses.

Cell culture
Colo-320, HCT-15, Caco-2, DLD-1, HCT-116, and SW-480 cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in humidified atmosphere containing 5% CO₂.

Reagents and antibodies
C-75, Cerulinen, direct AKT inhibitor, and zVAD-fmk inhibitor were purchased from Calbiochem (San Diego, CA). MTT and Bax 6A7 antibody was purchased from Sigma (St Louis MO, MA). Antibodies against phospho-AKT (Ser 473), phospho-FKHR, phospho-GSK3, cleaved caspase-3, and BID antibodies were purchased from Cell Signaling Technologies (Beverly, MA). FASN, Cytochrome c, β-actin, caspase-3, SKP-2, p27, and PARP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Caspase-8 antibody was purchased from R&D (USA). Annexin V kit was purchased from Molecular Probes (Eugene, OR). Apoptotic DNA-ladder kit was obtained from Roche (Penzberg, Germany).

Apoptotic assay
Cell lines were treated with and without C-75 for 24 and 48 h, and apoptosis was measured by annexin V/PI staining and DNA laddering using a 1.5% agarose gel as described earlier (30–32).

Cell lysis and immunoblotting
Cells were treated with C-75 as described in the legends and lysed as described earlier (33). Proteins were immunoblotted...
with different antibodies and visualized by the enhanced chemiluminescence (Amersham, Piscataway, NJ) method.

**Detection of Bax conformational changes**
This assay was performed as described earlier (31). Briefly, after treatment with indicated reagents for indicated time points, cells were harvested, washed with PBS, and lysed with Chaps lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps). Immunoblotting was performed using Bax polyclonal antibody.

**Measurement of mitochondrial potential and cytochrome c release**
After treatment of CRC cell lines with C-75 for 48 h, mitochondrial membrane potential was measured using JC1 dye and release of cytochrome c was analyzed using immunoblotting of mitochondrial and cytosolic protein fractions as described earlier (32,34).

**Gene silencing using siRNA**
FASN siRNA, AKT1 siRNA, AKT2 siRNA, and scrambled control siRNA were purchased from Qiagen (Valencia, CA). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h after which the lipid and siRNA complex was removed and fresh growth medium was added. Cells were lysed 48 h after transfection, and specific protein levels were determined by western blot analysis with specific antibodies.

**Animals and xenograft study**
Six-week-old NUDE mice were obtained from Jackson Laboratories (Maine) and maintained in a pathogen-free animal facility at least 1 week before use. All animal studies were done in accordance with institutional guidelines. For xenograft study, mice were inoculated subcutaneously into the right abdominal quadrant with 5 × 10^6 HCT-15 or Colo-320 cells in 200 μl PBS. After 1 week, mice were randomly assigned into three groups: two groups receiving two doses of C-75 (10 and 20 mg/kg) and remaining one group receiving 0.9% saline. After 1 week, mice were killed and individual tumors were weighed, then snap-frozen in liquid nitrogen for storage.

**RESULTS**

**FASN expression and its correlation with p-AKT and other clinicopathological parameters**
Elevated levels of FASN expression have been reported in a variety of solid tumors (7,20,36). We, therefore, first sought to study the expression of FASN and its association with different clinicopathological parameters in 448 cases of CRC in a TMA format. High levels of FASN expression were seen in 27.1% of the colorectal carcinomas (Figure 1). The incidence of expression for other immunohistochemistry markers is presented in Table 1. As shown in Table 1, FASN overexpression was not associated with age, gender, tumor site, American Joint Cancer Committee stage, and tumor differentiation. However, FASN overexpression was significantly associated with overexpression of p-AKT (P < 0.01) and Ki-67 (P < 0.01). FASN overexpression was also significantly associated with overexpression of SKP2 (P = 0.02). FASN overexpression was significantly more common in the MSI group as compared with MSS group (P < 0.01). FASN expression was significantly more common in the colorectal carcinoma subset with PI3K mutation as compared with colorectal carcinomas without PI3K mutations (P = 0.03). There was no significant association between expression of FASN and overall survival (P = 0.21). Finally, FASN overexpression was found to be higher in Middle Eastern CRC as compared with the Western data (11).

**C-75 causes apoptosis in a dose-dependent manner in CRC cell lines**
Our clinical data showed high level of FASN expression in CRC, and it has been shown earlier that FASN overexpression provides a selective advantage to tumor cells by promoting proliferation and inhibiting apoptosis (7,8,14). Interestingly, FASN inhibition has been shown to be more effective in inhibiting cell viability and initiating apoptosis (7,8,14). Therefore, we investigated whether C-75 treatment of CRC cell lines induced cell-cycle arrest or apoptosis in MSI and MSS cell lines. We selected two CRC cell lines, HCT-15, an MSI and Colo-320, an MSS. Both cell lines were treated with C-75 for 24 and 48 h and cell-cycle fractions were determined. As shown in Figure 2a, after 24 h treatment, there was substantial increase in the G2/M population in both cell lines. G2/M population increased from 16.16 ± 3.2% in untreated sample to 18.93 ± 4.1% at 25 μM treatment and 39.6 ± 3.9 after 50 μM treatment of C75 in Colo-320 cell line and from 11.01 ± 3.1% to 13.33 ± 4.2% and 36.17 ± 3.6% in HCT-15 cell line. Interestingly, after 48 h treatment, the sub-G1 population of cells increased in both the cell lines, from 3.23 ± 0.9% in untreated sample to 16.16 ± 3.1% at 25 μM and 27.92 ± 5.0% at 50 μM in Colo-320 cell line and from 6.10 ± 1.9% to 15.95 ± 3.2% and 59.95 ± 6.1% in HCT-15 cell line (Figure 2b). This increase in sub-G1 population was accompanied by loss in G0/G1, S, and G2/M phase in treated cells. We also used annexin V/PI dual staining and DNA laddering for confirmation of C-75-induced apoptosis in CRC cells. Cells were treated with 25 and 50 μM C-75 for 48 h and apoptosis was measured by annexin V/PI dual staining. As shown in Figure 2c, treatment of MSS Colo-320 cells with 25 and 50 μM induced 42 ± 6.2% and 53 ± 5.8% apoptosis, respectively. Interestingly, the response to C-75 treatment in MSI HCT-15 cell line was more pronounced at 50 μM dosage 34 ± 4.5% and 87 ± 10.3%. Similar results were obtained in two additional MSS cell lines, Caco-2 and SW-480, and two MSI cell lines, DLD-1 and HCT-116. As shown in Figure 2c (lower panel), the response of C-75 treatment was more pronounced in MSI cell lines as compared with MSS cell lines. We analyzed DNA fragmentation, which is another hallmark of apoptosis. As shown in Figure 2d, C-75 caused a dose-dependent DNA fragmentation in Colo-320 and HCT-15 cell lines. Finally, we averaged the percentage apoptosis of all three MSS cell lines and compared them with the
average of three MSI cell lines after treatment with C-75. As shown in Figure 2e, there was a statistically significant difference in the response of MSI cell lines as compared with MSS cell lines ($P < 0.01$). These data suggest that even though all the cell lines respond to C-75 inhibition, the apoptotic response is significantly higher in MSI cell lines.

**FASN-mediated regulation of PI3/AKT signaling pathways in CRC cell lines**

Our clinical data also suggests significant association between FASN and AKT; we, therefore, sought to determine the effect of FASN inhibition on AKT signaling pathway. Using the CRC cell lines, we sought to determine the expression of FASN and p-AKT and their response to C-75 treatment. As shown in Figure 3a, both the cell lines expressed constitutive FASN and p-AKT and treatment of CRC cells with C-75 suppressed FASN expression and dephosphorylated constitutively active AKT. C-75 treatment of CRC cell lines also inhibited constitutively phosphorylated FKHRL1 and GSK3, downstream targets of AKT pathway (Supplementary Figure 1). Recently, it has been shown that inhibition of FASN causes downregulation of SKP2, a component of an E3 ubiquitin ligase that
### Table 1. Correlation between FASN and clinicopathologic features

| Feature                        | High (3)   | Low (0–2) | Total number of cases | Gender | Tumor stage | PIK3CA mutation | MSI molecular |
|-------------------------------|------------|-----------|-----------------------|--------|-------------|-----------------|---------------|
|                               | n          | %         | n                     | n      | n           |                 |               |
| Total number of cases         | 403        |           | 109                   | 294    |             |                 |               |
| Age                           |            |           |                       |        |             |                 |               |
| ≤ 50 Years                    | 129        | 32.0      | 35                    | 94     | 27.1        |                 |               |
| > 50 Years                    | 274        | 68.0      | 74                    | 200    | 72.9        |                 |               |
| Gender                        |            |           |                       |        |             |                 |               |
| Male                          | 199        | 49.4      | 53                    | 146    | 26.6        |                 |               |
| Female                        | 204        | 50.6      | 56                    | 148    | 72.4        |                 |               |
| Tumor site                    |            |           |                       |        |             |                 |               |
| Left colon                    | 338        | 83.9      | 86                    | 252    | 74.6        |                 |               |
| Right colon                   | 65         | 16.1      | 23                    | 42     | 25.4        |                 |               |
| Histological type             |            |           |                       |        |             |                 |               |
| Adenocarcinoma                | 353        | 87.6      | 95                    | 258    | 73.1        |                 |               |
| Mucinous carcinoma            | 50         | 12.4      | 14                    | 36     | 28.0        |                 |               |
| Tumor stage*                  |            |           |                       |        |             |                 |               |
| I                             | 51         | 13.4      | 19                    | 32     | 37.2        |                 |               |
| II                            | 133        | 34.9      | 30                    | 103    | 22.6        |                 |               |
| III                           | 148        | 38.8      | 38                    | 110    | 25.7        |                 |               |
| IV                            | 49         | 12.9      | 14                    | 35     | 28.6        |                 |               |
| Differentiation               |            |           |                       |        |             |                 |               |
| Good                          | 31         | 7.7       | 6                     | 25     | 19.3        |                 |               |
| Moderate                      | 304        | 75.4      | 78                    | 226    | 25.7        |                 |               |
| Poor                          | 68         | 16.9      | 25                    | 43     | 36.8        |                 |               |
| p-AKT*                        |            |           |                       |        |             |                 |               |
| Negative (0–1)                | 107        | 27.4      | 14                    | 93     | 31.2        |                 |               |
| Positive (2–3)                | 283        | 72.6      | 94                    | 189    | 68.8        |                 |               |
| SKP2*                         |            |           |                       |        |             |                 |               |
| ≥ 50                          | 111        | 28.4      | 40                    | 71     | 36.0        |                 |               |
| < 50                          | 280        | 71.6      | 68                    | 212    | 64.0        |                 |               |
| p27kip1*                      |            |           |                       |        |             |                 |               |
| ≥ 50                          | 146        | 38.6      | 45                    | 101    | 30.8        |                 |               |
| < 50                          | 232        | 61.4      | 63                    | 169    | 69.2        |                 |               |
| Ki-67*                        |            |           |                       |        |             |                 |               |
| Positive                      | 337        | 86.9      | 105                   | 232    | 31.2        |                 |               |
| Negative                      | 51         | 13.1      | 2                     | 49     | 3.9         |                 |               |
| PIK3CA mutation*              |            |           |                       |        |             |                 |               |
| Absent                        | 331        | 86.7      | 85                    | 246    | 25.7        |                 |               |
| Present                       | 51         | 13.3      | 21                    | 30     | 41.2        |                 |               |
| MSI molecular*                |            |           |                       |        |             |                 |               |
| MSI-H                         | 74         | 20.0      | 31                    | 43     | 41.9        |                 |               |
| MSI-S/L                       | 298        | 80.0      | 71                    | 227    | 58.1        |                 |               |

*Data were available in 381 patients.

bP-AKT, SKP2, P27, Ki-67, PIK3CA mutation and MSI molecular data were not available in all 403 cases with FASN information. Analysis failure of the IHC markers tested ranged from 39 cases for p-AKT to 55 cases for p27kip1 and was attributed to missing spots or fixation artifacts.
tags p27Kip1 for degradation by proteasomes (37). As shown in Figure 3a, C-75 treatment of CRC cells downregulated the expression of SKP2 and elevated the expression of p27Kip1. These data suggest that FASN-mediated growth signaling involves AKT as well as proteasomal pathways in CRC cells. To further confirm whether AKT and SKP2 are actually involved in FASN pathway, we performed transfection studies with siRNA specifically targeted against FASN to determine the status of these proteins in CRC cell lines. HCT-15 cells were transfected with FASN-specific siRNA and like C-75, the siRNA-targeting FASN downregulated the expression of FASN protein, decreased phosphorylation of AKT, downregulated SKP2, and elevated expression of p27Kip1 (Figure 3b). Similar results were obtained for Colo-320 cell line after transfection with siRNA against FASN (data not shown). Treatment of CRC cell lines with Cerulenin, another inhibitor of FASN-induced apoptosis in a dose-dependent manner (Supplementary Figure 2), downregulated the expression of FASN (Supplementary Figure 3) and p-AKT (data not shown), as detected by western blotting supporting the role of FASN in the regulation of AKT signaling pathway.

To better understand the association between FASN and AKT, we performed transfection studies with siRNA against AKT. AKT-specific siRNA transfection in HCT-15 cell line did not show any effect on FASN expression but dephosphorylated AKT. AKT-specific siRNA transfection in HCT-15 cell line did not show any effect on FASN expression but dephosphorylated AKT. AKT was transfected with FASN-specific siRNA and like C-75, the siRNA-targeting FASN downregulated the expression of FASN protein, decreased phosphorylation of AKT, downregulated SKP2, and elevated expression of p27Kip1 (Figure 3c). Similar results were obtained for Colo-320 cell line after transfection with siRNA against FASN (data not shown). Treatment of CRC cell lines with Cerulenin, another inhibitor of FASN-induced apoptosis in a dose-dependent manner (Supplementary Figure 2), downregulated the expression of FASN (Supplementary Figure 3) and p-AKT (data not shown), as detected by western blotting supporting the role of FASN in the regulation of AKT signaling pathway.

To better understand the association between FASN and AKT, we performed transfection studies with siRNA against AKT. AKT-specific siRNA transfection in HCT-15 cell line did not show any effect on FASN expression but dephosphorylated AKT (Figure 3c). In addition, AKT inhibitor decreased AKT phosphorylation without affecting the FASN protein level in both the cell lines (Figure 3c). AKT inhibitor treatment also caused about 50% and 56% apoptosis in Colo-320 and HCT-15 cell line (Figure 3d). These results suggest that FASN is acting
upstream of AKT and AKT inhibition does not lead to change in FASN levels.

**C-75 treatment of CRC cells induced apoptosis via mitochondrial pathway- caspase mediated in CRC cell lines**

Next, we determined whether downregulation of FASN and p-AKT signaling involves mitochondria in CRC cell lines. C-75 treatment resulted in activation of caspase-8 leading to truncation of BID in both cell lines tested (**Figure 4a**), as inferred by decreased intensity of full-length BID band. As it is known that truncated BID plays a role in the activation of Bax, we examined Bax in response to C-75 treatment. As shown in **Figure 4b**, conformationally changed Bax was detected in both cell lines after C-75 treatment for 24h. We then tested the effect of C-75 on mitochondrial membrane potentials in these cells. As shown in **Figure 4c**, treatment of both cell lines with C-75 resulted in loss of mitochondrial membrane potential as measured by JC1 stained green fluorescence depicting apoptotic cells. We then studied the release of cytochrome c from mitochondria into cytosole. As shown in **Figure 4d**, higher level of cytochrome c was measured in cytosolic and lower levels in mitochondrial fraction in both cell lines after C-75 treatment. We then sought to determine whether C-75-induced release of cytochrome c is capable of activation of caspase-3 and PARP. **Figure 5a** shows that C-75 treatment resulted in the activation of caspase-3 and cleavage of PARP in Colo-320 and HCT-15 cells. In addition, pretreatment of CRC cells with 80 μM z-VAD-fmk, a universal inhibitor of caspases, followed by C-75 treatment, abrogated apoptosis from 50% to 12% in Colo-320 cells and from 59% to 9% in HCT-15 cells as well as prevented caspase-3 and PARP activation induced by C-75 (**Figure 5b** and **c**), clearly indicating that caspases play a critical role in C-75-induced apoptosis in CRC cells.

**Figure 3.** Effect of C-75 treatment on fatty acid synthase (FASN) and its downstream signaling pathways. (a) Colo-320 and HCT-15 cells were treated with and without 25 and 50-μM C-75 for 48h. After cell lysis, 20-μg proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against FASN, p-AKT-Ser 473, SKP-2, p27, and β-actin. Data shown are from a representative experiment repeated three times with similar results. (b) HCT-15 cells were transfected with scrambled siRNA and FASN siRNA (50 and 100nm). After 48h, cells were lysed and proteins were immunoblotted with antibodies against FASN, p-AKT-Ser473, SKP-2, p27, and β-actin. One of the three experiments is depicted in this figure. (c) Cells were transfected with either 100nm scrambled siRNA or 50 and 100nM pooled AKT1 and two siRNA for 48h. A measure of 20-μg proteins was immunoblotted with antibodies against p-AKT-Ser473, FASN, and β-actin. Each experiment was repeated twice to confirm reproducibility. (d) Colo-320 and HCT-15 cells were treated with and without 100μM direct AKT inhibitor for 48h, cells were either lysed and immunoblotted with antibodies against p-AKT-Ser473, FASN, and β-actin or stained with fluorescein-conjugated annexin V / PI and analyzed by flow cytometry.
**In vivo activity of FASN inhibitor C-75 against CRC cancer cells xenograft**

Our observation that CRC cells exhibit enhanced sensitivity to FASN inhibitor-induced apoptosis *in vitro* suggests the potential for therapeutic responses to treatment of CRC with FASN inhibitor *in vivo*. Therefore, ability of C-75 to inhibit CRC tumor growth was examined with a mouse xenograft model of CRC cancer. NUDE mice were inoculated subcutaneously in the right abdominal quadrant with 5 million HCT-15 cells. Mice were then treated with either two doses of C-75 treatment group (10 and 20 mg/kg/dose), or vehicle DMSO-treated control groups (n = 6). After 4 weeks of treatment, mice were killed and tumors were collected. As shown in Figure 6a, C-75 treatment causes a time-dependent regression of HCT-15 xenograft tumors in mice as compared with vehicle-treated mice. As shown in Figure 6a, at first week, tumor volume for vehicle-treated mice was 68.9 mm$^3$, 39.4 mm$^3$ for mice treated with 10 mg/kg C75, and 54.7 mm$^3$ for 20 mg/kg treatment. Second week measurements were 240, 98.6, and 122.6 mm$^3$, third week measurements were 729.1, 305, and 122.6 mm$^3$, and after 4 weeks of treatment, the tumor volume measurements were 1,210.7, 378.3, and 203.5 mm$^3$ in vehicle-treated mice, 10 mg/kg treatment, and 20 mg/kg treatment, respectively. The regression reached significance (P < 0.05) at the end of third week of treatment by C-75. A significant reduction in tumor weight (Figure 6b) was observed in mice treated with C-75.
DISCUSSION

In light of our earlier findings that alteration of PI3K/AKT pathway is frequently seen in large number of primary CRC specimens and considering the role of PI3K/AKT signaling pathway in regulating FASN expression (2,17–20), we sought to explore the potential link between FASN overexpression and PI3K/AKT pathway alteration in a large series of CRC sample and to study the effect of FASN inhibition on CRC both in vitro and in vivo. Our study showed a significant association between FASN overexpression and p-AKT expression in CRC, suggesting that FASN plays an important role in the pathogenesis of this molecular subtype of CRC.

We have reported earlier that activating mutation of PIK3CA is also seen mainly in MSI subtype of CRC samples (2). So the apparent association between FASN overexpression and (P < 0.05) as the weight of the tumor decreased from 2.51 g in vehicle-treated mice to 1.01 g in mice treated with 10 mg/kg C-75 and 0.84 g in mice treated with 20 mg/kg C-75. Additionally, images of tumor before and after necropsy showed that C-75 treatment resulted in shrinkage of tumor size (Figure 6c). As shown in Figure 6d, the level of FASN and p-AKT proteins were markedly decreased in primary tumors of mice treated with C-75 as compared with vehicle-treated mice as detected by western blotting. Immunohistochemistry done on HCT-15 xenograft tumor sections showed a reduced level of FASN as well as p-AKT staining after C-75 treatment (Figure 6e) that was statistically significant between untreated group vs. 20 mg/kg/dose C-75-treated group (P = 0.04 and P < 0.01). Finally, we also examined the effect of C-75 treatment in MSS Colo-320 cell line xenograft (Supplementary Figure 4). Our data suggests that MSS xenografts respond in a similar manner as MSI xenografts but with a slower rate.

Figure 5. Activation of caspases by C-75 treatment in CRC cell lines. (a) Colo-320 and HCT-15 cells were treated with and without C-75 for 48 h. Cells were lysed and proteins were immunoblotted with antibodies against pro-caspase-3, cleaved caspase-3, PARP, and β-actin. Each experiment was repeated three times to confirm reproducibility. (b) CRC cell lines were pretreated with either 80 μM z-VAD/fmk for 2 h and treated with 50 μM of C-75 for 48 h and analyzed by antibodies against pro-caspase-3, cleaved caspase-3, PARP, and β-actin or (c) apoptosis was measured by annexin V/PI staining. The results are expressed as mean ± s.d. (n = 3).
various molecular features (PIK3CA mutation and Ki67 proliferative marker) might be mediated through MSI. As MSI status reflects genomic aberrations in tumor cells and determine molecular subtypes of CRC cancer, the clinicopathological features and FASN expression are likely influenced by genomic status of tumor cells. Our findings are in partial agreement with earlier study where close clinical association was seen between FASN overexpression and MSI status (11). Although FASN expression has been shown to be a prognostic marker in certain cancer ((38–41), FASN expression was not found to have a prognostic value in our study.

To further elucidate the pivotal role of FASN expression in determining the molecular characteristic of CRC, we conducted in vitro analysis on two cell lines that were screened earlier for mismatch repair deficiency and activating PIK3CA mutation, HCT-15 MSI, PIK3CA mutation +ve, and Colo-320 MSS, PIK3CA mutation –ve. Our in vitro studies further support the correlation between FASN expression and AKT activation in both MSI and MSS CRC cell lines. Interestingly, inhibition of FASN also

**Figure 6.** C-75 inhibits growth of HCT-15 xenograft and downregulates fatty acid synthase (FASN) and inactivates AKT in vivo. NUDE mice at 6 weeks of age were injected s.c. with 5 million HCT-15 cells. (a) Effect of C75 on HCT-15 xenograft. The volume of each tumor was measured every week. The average (n=6) tumor volume in vehicle-treated control mice and treated with C-75 was plotted. The results are expressed as mean±s.d. (n=6) (*P<0.05). (b) After 4 weeks of treatment, mice were killed and tumor weights were measured. The results are expressed as mean±s.d. *P<0.05 compared with vehicle-treated mice by Student’s t-test. (c) Representative tumor images of vehicle and C-75-treated mice before and after necropsy. (d) Whole cell homogenates from mice treated with vehicle (1–2), C-75 (10 mg/kg) (3–4), and C-75 (20 mg/kg) (5–6) were immunoblotted with FASN, p-AKT, and actin antibodies. (e) Paraffin-embedded tumor tissues from mice were stained for FASN and p-AKT by immunohistochemistry, and a significant reduction in FASN and p-AKT staining was seen between vehicle-treated mice and mice treated with C-75 at 20 mg/kg/dose.
substantially reduced levels of SKP2, an F-box protein essential for proteasome degradation of p27kip1. Consequently, we conclude that inhibition of FASN acts upstream of proteasome to control p27kip1 levels and ultimately blocks cell-cycle progression. The mechanistic connection between the FASN blockade and reduction in SKP2 might be mediated through AKT activation.

Recently, it has been shown that AKT modulates the expression of FASN in a positive feedback manner in ovarian cancer cells (20). Our pharmacological inhibition and gene silencing studies suggests that inhibition of AKT does not affect the expression of FASN. On the other hand, C-75 treatment of CRC cell lines as well as gene silencing of FASN inactivated AKT activity in addition to downregulation of FASN expression in both the CRC cell lines. These findings suggest that FASN is an upstream effector of AKT in CRC cell lines.

Our in vivo studies of the affect of C-75 on HCT-15 and Colo-320 tumor growth in a murine xenograft model are consistent with results obtained with breast cancer, ovarian cancer, and mesothelioma xenograft models (14,20,42). In addition to an overall significant inhibition of xenograft tumor growth, western blot analysis of tumors showed decreased AKT activity in most C-75-treated mice, concordant with reduced FASN protein level. Thus, FASN inhibition may have significant promise as a therapeutic approach in subset of CRC.

In conclusion, our findings suggest that there is an overall increase in expression of FASN in CRC tumors of this region (11). Therefore, targeting FASN for treatment of CRC maybe more beneficial in CRC tumors. Furthermore, data presented here demonstrate a significant correlation between expression of FASN and active AKT in CRC and indicate that inhibition of PI3K/AKT signaling synergize the FASN inhibitors to induce apoptosis in CRC cell lines with constitutively active AKT. This may have significant clinical implications. Elucidating the molecular link between FASN overexpression and AKT activation may be important as a biomarker and may serve as promising target for therapeutic intervention in this molecular distinct subtype of CRC.

**CONFLICT OF INTEREST**

**Guarantor of the article:** Khawla S. Al-Kuraya, MD, FCAP.

**Specific author contributions:** Shahab Uddin, PhD: designed the study, performed experiments, analyzed data, wrote the paper, and gave final approval for submission of the manuscript in its current format. Azhar Hussain, MBBS: designed the study, performed experiments, analyzed data, helped in writing and proofreading the manuscript, and gave final approval for submission of the manuscript in its current format. Maqbool Ahmed, PhD: performed experiments, analyzed the data, helped in drafting the manuscript, and gave final approval for submission of the manuscript in its current format. Jehad Abubaker, PhD: performed experiments, analyzed the data, and gave final approval for submission of the manuscript in its current format. Nasser Al-Sanea, MD: contributed substantially to the acquisition and interpretation of clinical data, participated in revising and formulating the content of the manuscript and gave final approval for submission of the manuscript in its current format. Alaa Abdul Jabbar, MD: contributed substantially to the acquisition and interpretation of clinical data, participated in revising the content of the manuscript, and gave final approval for submission of the manuscript in its current format. Luai H Ashari, MD: contributed substantially to the acquisition and interpretation of clinical data, and gave final approval for submission of the manuscript in its current format. Sama Al-Homoud, MD: contributed substantially to the acquisition and interpretation of clinical data, participated in revising and formulating the content of the manuscript and gave final approval for submission of the manuscript in its current format. Fouad Al-Dayel, MD: contributed substantially to the acquisition and interpretation of clinical data and gave final approval for submission of the manuscript in its current format. Prashant Bavi, MD: designed the study, performed experiments, analyzed data, helped in writing the manuscript, performed statistical analysis, and gave final approval to submit the manuscript in its present form. Khawla S. Al-Kuraya, MD, FCAP: designed, formulated, and lead the project, analyzed and interpreted data, wrote the manuscript, and gave final approval.

**Potential competing interests:** None. (This work was not supported by the National Institutes of Health, the Wellcome Trust, the Howard Hughes Medical Institute, or others.)

**Financial support:** None.

**ACKNOWLEDGMENTS**

We thank Saeeda Ahmed, Azadali Moorji, Valerie Atizado, Hasan Al-Dossari, and Valorie Balde for their technical assistance and Sriram Devarajan for clinical data analysis.

**Study Highlights**

| WHAT IS CURRENT KNOWLEDGE |
|----------------------------|
| 🔄 Human epithelial cancers express high levels of fatty acid synthase (FASN). |
| 🔄 FASN is linked with activation of phosphatidylinositol-3’-kinase (PI3K)/AKT pathway. |
| 🔄 Advances in the understanding of tumor biology have lead to the development of targeted therapies. |
| 🔄 One of the promising targets for therapeutic intervention is FASN. |

| WHAT IS NEW HERE |
|------------------|
| 🔄 High levels of FASN expression are seen in 27.1% of the colorectal carcinomas in the Middle Eastern origin. |
| 🔄 Inhibition of FASN with C-75 treatment induces cell-cycle arrest and apoptosis in colorectal cancer (CRC) cell lines. |
| 🔄 C-75 treatment of CRC cell lines suppresses FASN expression and dephosphorylates constitutively active AKT. |
| 🔄 C75 treatment causes conformational changes of Bax protein. |
| 🔄 C75 treatment causes activation of mitochondrial apoptotic pathway and induces caspase-dependent apoptosis. |
| 🔄 The findings in this study suggest that there is an overall increase in expression of FASN in CRC tumors. Targeting FASN as a therapeutic intervention may be an attractive strategy for the treatment of CRC. |
REFERENCES

1. Watanabe T, Kobunai T, Toda E et al. Distal colorectal cancers with microsatellite instability (MSI) display distinct gene expression profiles that are different from proximal MSI cancers. Cancer Res 2006;66:9804–8.

2. Abubaker J, Bavi P, Al-Harbi S et al. Clinicopathological analysis of colorectal cancers with PIK3CA mutations in Middle Eastern population. Oncogene 2008;27:3539–45.

3. Douillard JY, Cunningham D, Roth AD et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. Lancet 2000;355:1041–7.

4. Khamly K, Jefford M, Michael M et al. Beyond 5-fluorouracil: new horizons in systemic therapy for advanced colorectal cancer. Expert Opin Investig Drugs 2005;14:607–28.

5. Hurwitz HI, Fehrenbacher L, Hainsworth JD et al. Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. J Clin Oncol 2005;23:3502–8.

6. Semenovich CF. Regulation of fatty acid synthase (FASN). Prog Lipid Res 1997;36:43–53.

7. Kuhajda FP. Fatty acid synthase and cancer: new application of an old pathway. Cancer Res 2006;66:5977–80.

8. Rossi S, Graner E, Febo P et al. Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. Mol Cancer Res 2003;1:707–15.

9. Aló PL, Visca P, Marci A et al. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. Cancer 1996;77:474–82.

10. Gansler TS, Hardman W III, Hunt DA et al. Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. Hum Pathol 1997;28:686–92.

11. Ogino S, Kawasaki T, Ogawa A et al. Fatty acid synthase over-expression in colorectal cancer is associated with microsatellite instability, independent of CpG island methylator phenotype. Hum Pathol 2007;38:842–9.

12. Wang YY, Kuhajda FP, Cheng P et al. A new model ELISA, based on two monoclonal antibodies, for quantification of fatty acid synthase. J Immunol 2002;169:3279–92.

13. Pizer ES, Pflug BR, Bova GS et al. Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. Prostate 2001;47:102–10.

14. Pizer ES, Thupari J, Han WF et al. Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. Cancer Res 2000;60:213–8.

15. DeSrichive E, Brusselmans K, Heyns W et al. RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. Cancer Res 2003;63:3799–804.

16. Pizer ES, Wood FD, Heine HS et al. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res 1996;56:1189–93.

17. Bandyopadhyay S, Pai SK, Watabe M et al. FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. Oncogene 2005;24:5389–95.

18. Porstmann T, Griffiths B, Chung YL et al. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene 2005;24:465–81.

19. Yang YA, Han WF, Morin PJ et al. Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Exp Cell Res 2002;279:80–90.

20. Wang HQ, Altomare DA, Skele KL et al. Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene 2005;24:3574–82.

21. Franke TF, Hornik CP, Segev I et al. PI3K/Akt and apoptosis: size matters. Oncogene 2003;22:8983–98.

22. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.

23. Reichert M, Saur D, Hamacher B et al. Phosphoinositide-3-kinase signaling controls 5-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells. Cancer Res 2007;67:4149–56.

24. Häusler P, Popoff G, Eramo A et al. Protection of CD95-mediated apoptosis by activation of phosphatidylinositol 3-kinase and protein kinase B. Eur J Immunol 1998;1:57–69.

25. Di Cristofano A, Kotsi P, Peng YF et al. Impaired Fas response and autoimmunity in Pten−/− mice. Science 1999;285:2122–5.

26. Bavi P, Jehan Z, Atiziano V et al. Prevalence of fragile histidine triad expression in tumors from Saudi Arabia: a tissue microarray analysis. Cancer Epidemiol Biomarkers Prev 2006;15:1708–18.

27. Siraj AK, Bavi P, Abubaker JA et al. Genome-Wide expression analysis of Middle Eastern papillary thyroid cancer reveals c-met as a novel target for cancer therapy. J Pathol 2007;213:190–9.

28. Uddin S, Hussain AR, Siraj AK et al. Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival. Blood 2006;108:4178–86.

29. Shapira M, Ben-Izhak O, Bishara B et al. Alterations in the expression of the cell cycle regulatory protein cyclin kinase subunit 1 in colorectal carcinoma. Cancer 2004;9:1615–21.

30. Hussain AR, Al-Rasheed M, Manogaran PS et al. Curcumin induces apoptosis via inhibition of PI3'-kinase/AKT pathway in acute T cell leukemias. Apoptosis 2006;11:245–54.

31. Hussain AR, Al-Jomah NA, Siraj AK et al. Sanguinarine-dependent induction of apoptosis in primary effusion lymphoma cells. Cancer Res 2007;67:3888–97.

32. Uddin S, Hussain A, Al-Hussein K et al. Inhibition of phosphatidylinositol 3'-kinase induces preferentially killing of PTEN-null T leukemias through AKT pathway. Biochem Biophys Res Commun 2004;320:932–8.

33. Uddin S, Hussain AR, Al-Hussein KA et al. Inhibition of phosphatidylinositol 3'-kinase/AKT signaling promotes apoptosis of primary effusion lymphoma cells. Clin Cancer Res 2005;11:3102–8.

34. Uddin S, Hussain AR, Manogaran PS et al. Curcumin suppresses growth and induces apoptosis in primary effusion lymphoma. Oncogene 2005;24:7022–30.

35. Bazzaro M, Lee MK, Zoso A et al. Ubiquitin-proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. Cancer Res 2006;66:3734–63.

36. Milgraum LZ, Witters LA, Pasterнак GR et al. Enzymes of the fatty acid synthesis pathway are highly expressed in situ breast carcinoma. Clin Cancer Res 1997;3:2115–20.

37. Knowles LM, Axell F, Browne CD et al. Fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating Skp2. J Biol Chem 2004;279:30450–5.

38. McCarthy AD, Hardie DG. Fatty acid synthase: an example of protein evolution by gene fusion. Trends Biochem Sci 1984;9:60–3.

39. Cho KR, Vogelstein B. Genetic alterations in the adenoma–cancer sequence. Cancer 1992;70:1727–31.

40. Hamilton SR. The molecular genetics of colorectal neoplasia. Gastroenterology 1993;105:3–7.

41. Nishisho I, Nakamura Y, Miyoshi Y et al. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 1991;253:665–9.

42.Gabrielson EW, Pinn ML, Testa JR et al. Increased fatty acid synthase is a therapeutic target in mesothelioma. Clin Cancer Res 2001;7:153–7.