Accepted Manuscript

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PII: S0304-3835(18)30559-7

DOI: [10.1016/j.canlet.2018.09.006](https://doi.org/10.1016/j.canlet.2018.09.006)

Reference: CAN 14049

To appear in: *Cancer Letters*

Received Date: 8 June 2018

Revised Date: 3 August 2018

Accepted Date: 2 September 2018

Please cite this article as: P. Yang, C. Su, X. Luo, H. Zeng, L. Zhao, L. Wei, X. Zhang, Z. Varghese, J.F. Moorhead, Y. Chen, X.Z Ruan, Dietary oleic acid-induced CD36 promotes cervical cancer cell growth and metastasis via up-regulation Src/ERK pathway, *Cancer Letters* (2018), doi: 10.1016/j.canlet.2018.09.006.

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Abstract:

Epidemiological and experimental studies have revealed strong associations between dietary lipids and cancer risk. However, the molecular mechanisms underlying the effects of dietary fatty acids on the genesis and progression of cancer have been poorly explored. In this study, we found that a high olive oil diet stimulated cervical cancer (CC) carcinogenesis, and oleic acid (OA), the main lipid in olive oil, was associated with increased malignancy in HeLa cells. OA up-regulated the expression of CD36, which is the best characterized fatty acid transporter. Inhibiting CD36 prevented the tumor-promoting effects of OA, while overexpressing CD36 mimicked the effects of OA. Clinically, CD36 expression was positively correlated with tumor progression and poor prognosis in patients with CC. Furthermore, OA induced Src kinase and downstream ERK1/2 pathway activation in a CD36-dependent manner. Pretreatment of HeLa cells with an Src kinase inhibitor largely blocked the tumor-promoting effect of OA. Our findings suggest that dietary OA exerts a stimulatory effect on CC growth and metastasis, and CD36 might be a promising therapeutic target that acts against CC through an Src/ERK-dependent signaling pathway.

Dietary oleic acid-induced CD36 promotes cervical cancer cell growth and metastasis via up-regulation Src/ERK pathway

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Declarations of interest: none

Abstract:

Epidemiological and experimental studies have revealed strong associations between dietary lipids and cancer risk. However, the molecular mechanisms underlying the effects of dietary fatty acids on the genesis and progression of cancer have been poorly explored. In this study, we found that a high olive oil diet stimulated cervical cancer (CC) carcinogenesis, and oleic acid (OA), the main lipid in olive oil, was associated with increased malignancy in HeLa cells. OA up-regulated the expression of CD36, which is the best characterized fatty acid transporter. Inhibiting CD36 prevented the tumor-promoting effects of OA, while overexpressing CD36 mimicked the effects of OA. Clinically, CD36 expression was positively correlated with tumor progression and poor prognosis in patients with CC. Furthermore, OA induced Src kinase and downstream ERK1/2 pathway activation in a CD36-dependent manner. Pretreatment of HeLa cells with an Src kinase inhibitor largely blocked the tumor-promoting effect of OA. Our findings suggest that dietary OA exerts a stimulatory effect on CC growth and metastasis, and CD36 might be a promising therapeutic target that acts against CC through an Src/ERK1/2-dependent signaling pathway.

Keywords: high olive oil diet; fatty acid transporter; cell proliferation; cell migration; tyrosine kinase

1. Introduction

Cervical cancer (CC) is the second-most common female-specific carcinoma after breast cancer and accounts for approximately 8% of total cancer deaths in women worldwide [1]. CC, especially cervical adenocarcinoma, has a poor prognosis, with 5-year survival rates of only 30-40% or less for women with advanced-stage cancer [2]. Human papilloma virus (HPV) infection is the greatest risk factor for CC; however, many people with HPV infection do not develop CC, suggesting that additional factors are required for the induction and progression of CC. Several other contributing factors, including smoking, a weak immune system, and oral contraceptives, have been implicated, but not all of the factors are known.

In recent years, numerous epidemiologic studies have found that obesity, overweight, and serum lipid levels are risk factors for CC morbidity and mortality; these findings suggest that lipids are significantly associated with CC [3-5]. Dietary lipids, major nutritional components, are important determinants associated with the risk of cancer development. Nonetheless, human data regarding the association between lipid intake and cancer are conflicting, mainly depending on the type and quantity of lipids. High saturated fatty acid intake, mainly from animal sources, could increase cancer risk, especially breast cancer [6]. Polyunsaturated fatty acids, especially eicosapentaenoic acid and docosahexaenoic acid from fish oil, inhibit breast and colon tumor growth and metastasis [7, 8]. However, the role of monounsaturated fatty acids, primarily oleic acid (OA) (18:1 n-9) and its main dietary source, olive oil, in cancer development remain unclear. Experimental studies

addressing the effects of olive oil on cancer progression have been conducted mainly in breast cancer models, and olive oil seems to have protective effects [7, 9]. However, inconsistent data have also been reported, which showed a tumor-enhancing role of OA in many cancer types [10-13]. So far, the role of OA in cancer is uncertain and has attracted much attention in recent years.

It is well known that cells can take up fatty acid by passive diffusion and by receptor-mediated mechanisms involving several fatty acid transporters, of which the fatty acid translocase CD36 is the best characterized [14]. CD36 is an integral transmembrane glycoprotein expressed in various tissues, where it is involved in high-affinity uptake of long-chain fatty acids (LCFAs), mainly oleate and palmitate [15]. CD36 expression is strongly induced by LCFAs, which, in turn, mediate lipid metabolism and may also initiate signal transduction. There is increasing evidence that alterations in lipid metabolism are strongly associated with tumorigenesis; these alterations can regulate cancer cell proliferation, differentiation, metastasis and survival [16]. A possible emerging role of CD36 in cancer has been proposed recently. Glioblastoma stem cells with high CD36 expression can enhance self-renewal and tumor initiation capacity [17]. A subpopulation of leukemic cancer stem cells with CD36-positive expression was shown to have unique metabolic properties and evade chemotherapy [18]. CD36⁺ oral carcinoma cells were unique in their ability to initiate metastasis relying on changes in lipid metabolism [19].

In this study, we sought to determine the effects of a high fat diet enriched with olive oil on the development of experimental CC and explore the underlying

molecular mechanisms. High olive oil diet feeding enhanced CC progression, and then we examined the regulation of CD36 by OA. Furthermore, we explored the role of CD36 and its downstream signaling pathways in OA-induced tumor growth. Based on this study, we suggest that CD36 might be a therapeutic target for CC patients with lipid disorders.

2. Materials and methods

2.1 Animal models

Animal care and experimental procedures were performed with approval from the Animal Care Committee of Chongqing Medical University. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals. Four-week-old BALB/c-nu/nu nude mice were assigned randomly to receive a normal diet (10% kcal from fat) or a high olive oil diet (45% kcal from fat) purchased from Htpharma Technology Development Co., Ltd. (Beijing, China). The mice were inoculated subcutaneously in their left flanks with 5×10^6 HeLa cells. Tumor growth was measured every 3 days, and the volumes of the xenograft tumors were calculated using the following standard formula: length $\times$ width $\times$ width $\times$ 0.5. In another experiment, HeLa cells were injected into the mice via their tail veins (1×10^6 cells) to establish a metastatic model as described previously [20].

2.2 Cell culture

HeLa cells were cultured in high glucose DMEM containing 10% fetal bovine serum (FBS). HeLa cell line was authenticated by short tandem repeat analysis. The

CD36 overexpression (CD36OE) stable cell line was constructed by transfection with a recombinant lentivirus (Ubi-MCS-3FLAG-SV40-puromycin) containing CD36 cDNA or an empty vector as a control, while the CD36 knockdown (siCD36) cell line was established by transfection with a CD36 shRNA lentiviral construct (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) targeting 5'-GGCTGTGTTTGGAGGTATTCT-3' or a scrambled shRNA lentivirus as a control. The transfected cells were then selected with puromycin. All lentiviruses were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China).

2.3 Cell proliferation assay

HeLa cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h, the cells were incubated in serum-free medium for 12 h. Then, the cells were subjected to OA loading (from 0 to 100 μ M) for different times. All experiments were carried out in serum-free DMEM medium containing 0.2% fatty acid-free BSA. The OD values were measured at 450 nm after incubation with CCK-8 reagent for 2 h at 37°C.

2.4 Cell cycle analysis

HeLa cells were treated with or without OA for 48 h. Then, the cell cycle analysis was performed using flow cytometry after RNase A treatment and PI staining.

2.5 Transwell assays

For the transwell migration assays, HeLa cells in the upper chamber were treated with or without different concentrations of OA, while DME containing 10% FBS was added to the lower chambers. For the transwell invasion assays, the upper

membrane was coated with 40 μ l Matrigel (BD Biosciences) in advance. After incubation, the cells were fixed and stained with trypan blue.

2.6 Wound healing

HeLa cells were seeded in 24-well plates, and the monolayer was scratched with a pipette tip. After that, the cells were treated with or without OA for 0-72 h. Then, the wound areas were quantified using Image J software.

2.7 Colony formation assay

HeLa cells were plated in 6-well plates at a density of 4000 cells/well with medium containing 10% FBS. Then, the cells were treated with or without OA (5 μ M) for 2 weeks. Colonies were fixed and stained with a 0.1% crystal violet solution and counted grossly.

2.8 Histology and immunohistochemistry (IHC) analysis

HE staining and IHC analysis have been described previously [21]. The following primary antibodies were used: anti-CD36 (1:800, Novus), anti-PCNA (1:8000, CST), anti-vimentin (1:100, CST) and anti-E-cadherin (1:500, CST).

2.9 Real-time quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Takara) and reverse transcribed into cDNA. Next, the cDNA products were subjected to 2-step PCR amplification. The relative expression of the genes was analyzed using the $2^{-\Delta\Delta C_t}$ method, and β -actin was used as the internal reference gene.

2.10 Western blot analysis

Total protein was extracted using RIPA lysis buffer. Western blotting was

performed as previously described [21]. The following primary antibodies were used: anti-JNK, anti-P-JNK, anti-Src, anti-P-Src, anti-ERK, anti-P-ERK, anti-AKT, anti-P-AKT, anti-AMPK, and anti-P-AMPK (1:1000, CST); anti-CD36 (1:2000, Novus); and anti- β -actin (1:5000, Bioss). The protein bands were semi-quantified by ImageJ software.

2.11 Statistical analysis

CC clinical data were downloaded from The Cancer Genome Atlas (TCGA) database. The chi-square test was applied to determine the association between CD36 expression and the CC clinicopathological parameters. A survival analysis was conducted to compare the overall survival rates using Kaplan-Meier survival curves with log-rank tests.

Statistical analyses were performed using Student's *t* test when only two groups were compared, and one-way analysis of variance followed by Turkey's multiple comparison test was used for three groups. All data are presented as the mean $\pm$ SEM, and *P* < 0.05 was considered significant.

3. Results

3.1 High olive oil diet aggravates CC growth and metastasis in a mouse xenograft model

Mice were divided into two groups and fed either a normal chow diet (10% kcal from fat) or a high olive oil diet (45% kcal from fat), and tumor growth was monitored for 40 days. Mice in the olive oil diet group had considerably greater tumor

growth than the control group, which included 1 mouse with no tumor (Fig. 1 A). Consistently, both the sizes and weights of the xenograft tumors were increased more than 6-fold in the olive oil diet-fed mice at the end of the experiment (Fig. 1 B, C). As the primary tumor did not metastasize, we established a metastasis model by injecting HeLa cells into the tail veins of the mice. Approximately 40 days later, the mice started to lose weight and were sacrificed. We observed the formation of tumor metastases in only the liver, while other distant metastases were not observed. Mice in the olive oil diet group had a higher metastasis incidence (4 in 10 mice) than mice in the normal group (1 in 11 mice) (Fig. 1 D). Significant increases in the size of the metastatic nodules were also found in mice fed the olive oil diet (Fig. 1 E).

3.2 OA promotes cell proliferation and migration

As the main component of olive oil is OA (up to 83%), we tested the modulation of cell function by OA in vitro. Cell viability and proliferation were analyzed using CCK-8 assays. No pharmaceutical toxicity was observed for 50 μ M OA treatment (Fig. 2 A), and low concentrations of OA stimulated HeLa cell proliferation in a dose- and time-dependent manner (Fig. 2B, C). A cell cycle analysis showed that the percentage of cells in S phase was increased, and that of cells in G2 phase was decreased by OA treatment (Fig. 2 D). Additionally, the OA-treated cells formed a higher number of colonies than the control cells (Fig. 2 E), which revealed the improved survival and proliferative capacity of OA-incubated cells. In addition, cell migration and invasion ability were also significantly increased by OA in a dose-dependent manner (Fig. 2 F, G). These data demonstrated that OA has

tumor-promoting effects in vitro.

3.3 CD36 expression was positively correlated with tumor progression

The xenograft tumors were then subjected to IHC analysis. We found significantly more PCNA-positive tumor cells, accompanied by higher CD36-membrane expression, in the tumors from the olive oil diet-fed mice than in those from the control mice (Fig. 3 A). Meanwhile, the mRNA and total protein expression of CD36 was increased by high olive oil diet feeding (Fig. 3 B, C). In vitro, OA treatment also elevated total and membrane expression of CD36 (Fig. 3 D, E and Supplemental Fig. S1A). To determine whether CD36 exerts a role in the development of CC, we analyzed publicly available data from patients with CC in TCGA database. First, we found that *CD36* expression was markedly increased in CC patients with an advanced tumor clinic stage, T stage and N stage (Fig. 3 F-H). Then, the chi-square test was used to evaluate the association between *CD36* expression and the clinicopathological parameters of CC patients. As shown in supplemental Table S1, *CD36* expression was positively correlated with the clinical stage and T stage of CC. Furthermore, high *CD36* expression was associated with a high risk of poor prognosis (HR=1.890, 95% CI 1.051 to 3.398) (Fig. 3 I). These data suggest that CD36 may participate in the pathogenesis of CC progression.

3.4 CD36 overexpression promotes tumor growth and metastasis in vitro and in vivo

To determine the effects of CD36 on experimental tumor growth, we constructed a stable HeLa cell line with CD36 overexpression that was confirmed by Western blot

analysis. (Supplemental Fig. S1B,C). As shown in Fig. 4 A-C, CD36 overexpression increased cell proliferation and migration similar to OA. Consistent with the in vitro results, CD36 overexpression enhanced subcutaneous xenograft tumor growth in mice (Fig. 4 D-F). The IHC examination revealed that higher CD36 expression was associated with tumor cell proliferation and invasion, as evidenced by increased PCNA and vimentin expression and decreased E-cadherin expression (Fig. 4 G, H). These experimental data confirmed the role of CD36 in tumor progression.

3.5 CD36 suppression blocks the tumor-stimulating effects of OA

Sulfo-N-succinimidyl oleate (SSO), an analogue of OA, specifically and irreversibly binds to CD36 and inhibits fatty acid uptake by CD36. We next determined the effects of SSO on cellular function. As expected, SSO pretreatment attenuated the cell proliferation and migration that was induced by OA (Fig. 5A-D). Then, we silenced the expression of CD36 by shRNA lentiviral transfection and the efficiency of CD36 knockdown was analyzed by western blot (Supplemental Fig. S1D). Similarly, knockdown of CD36 reversed OA-induced cell migration, invasion and proliferation (Fig. 5E, F and Supplemental Fig. S1E). These results indicate that the tumor-stimulating effects of OA may act through CD36-mediated signal transduction.

3.6 The CD36/Src/ERK pathway is involved in the tumor-promoting effects of OA

CD36 is a multi-functional protein that participates in a variety of signal transduction pathways associated with Src family kinases, JNK, and AMPK.

Therefore, we determined whether OA-induced CD36 activates these pathways. Interestingly, the tumors from olive oil-fed mice had marked Src tyrosine kinase activation, but no changes in the JNK or AMPK pathway (Fig. 6A). CD36 overexpression also induced Src phosphorylation both in cells and xenograft tumors (Fig. 6B). Regarding the downstream effectors of Src, olive oil diet feeding dramatically increased phosphorylation of ERK1/2, while not affecting AKT. In vitro, OA treatment activated the phosphorylation of Src and ERK1/2; on the contrary, CD36 knockdown suppressed the Src/ERK1/2 signaling pathway (Fig. 6B, C). Then, experiments were carried out to determine whether CD36-mediated Src activation plays a key role in the tumor-promoting function of OA. Pharmacological inhibition of Src function with SU6656 effectively prevented Src/ERK1/2 signal (Supplemental Fig. S1F) and completely blocked the effects of OA on cell proliferation, migration, and invasion (Fig. 6D, E). In addition, knockdown of CD36 failed to inhibit the proliferation and migration of SU6656-treated cells (Fig. 6F). These results suggest OA-mediated CD36 involved in tumor pathogenesis through the Src/ERK1/2 pathway.

4. Discussion:

The Mediterranean diet, characterized by a high consumption of olive oil, which is considered on top of the list of “nutraceutical”, provides health benefit effects especially by reducing major cardiovascular risk events [22, 23]. There has been growing interests regarding the possible role of olive oil in cancer prevention and

treatment. Although a large number of human studies have shown increased fat intake is positively associated with cancer risk, the Mediterranean diet seems to have protective effects [24]. Numerous epidemiological studies have suggested a favorable effect of the Mediterranean diet on cancer morbidity reduction, especially breast cancer and colon cancer [7, 25]. In addition, prospective, cohort, and epidemiological studies have shown an inverse association between dietary monounsaturated fatty acids, such as OA, and the risk of cancer, including breast and liver cancer [26, 27]. However, other studies have generated conflicting results, rendering the human studies on the effects of dietary olive oil or OA on cancer inconclusive [6, 27-30]. These conflicting results may be explained partly by the complexity of the interactions between genetic and environmental factors. However, few experimental studies have addressed the role of olive oil and OA in cancer genesis, and many questions remain to be explored. In the present study, a high olive oil diet did not protect nude mice from CC xenograft growth and metastasis; rather, this diet had a tumor-enhancing effect. Similarly, OA stimulated HeLa cell proliferation, migration, and invasion in vitro. Our results are consistent with the study from Vinciguerra *et al.*, which showed a positive association between dietary OA and hepatoma progression.

OA is a functional molecule that exerts a variety of effects on cell growth, cell proliferation, epithelial to mesenchymal transition, cell migration, and angiogenesis. Cancer cells rely mainly on fatty acids for membrane proliferation, energy storage, and signaling molecule generation. In addition, fatty acids influence cancer development by modulating signaling pathways involved in cell transformation and

tumorigenesis [7]. It has been widely described that fatty acids can directly bind to various nuclear receptors (LXR, PPAR and RXR) to activate their target gene transcription. Furthermore, fatty acids, such as OA, can activate membrane receptors, e.g. epidermal growth factor receptor (EGFR) and GPR40 proteins, which are critical regulators of mitogenic cell signaling [31, 32]. In addition, OA can modulate the activity of PKC, AMPK, MMP9 and PLC, as well as the gene expression of Her-2/neu and PTEN, which are involved in carcinogenesis [10, 33, 34]. Here, we showed a novel regulatory role of OA in up-regulating the mRNA and protein expression of the membrane receptor CD36, which may be critical for the OA-mediated tumor-enhancing effects.

Metabolic reprogramming has been recognized as a new hallmark of tumorigenesis. Metabolomics screening of CC patients has identified systemic changes in lipid metabolites, indicating a potential link between lipid metabolism and CC development [35, 36]. Deregulation of lipid metabolism can affect numerous cellular processes, including proliferation, survival, and differentiation of cancer cells [37]. Increased *de novo* fatty acid synthesis has emerged as a defining feature of cancer cells and has become an attractive cancer target [38, 39]. Aside from *de novo* synthesis, cancer cells may uptake fatty acids actively from the environment to sustain cell division and proliferation [39]. The fatty acid translocase CD36 is the best-characterized protein that mediates fatty acid uptake across the plasma membrane. CD36, which is highly expressed in metastatic ovarian tumors, scavenges LCFA from neighboring adipocytes to sustain rapid tumor growth and metastasis [40]. The

anti-proliferation effect of breast cancer cells by SCD1 inhibitor can be reversed by exogenous OA in a CD36-dependent pathway [41]. Additionally, CD36⁺ cancer stem cells, which have unique metabolic properties, have shown self-renewal, tumor initiation, chemotherapy resistance and metastatic activity [17-19]. In our study, CD36 overexpression in HeLa cells aggravated tumor growth and invasion in a xenograft mouse model; on the other hand, depriving HeLa cells of exogenous OA using CD36 inhibitors and siRNA knockdown of CD36 prevented the development of a malignant phenotype. Clinically, high CD36 expression was correlated with tumor progression and poor prognosis in CC patients. Taken together, our study and others suggest that the fatty acid transport protein CD36 may be a promising new target for antitumor therapy.

The tumor-promoting phenotype induced by OA that we observed in our study may rely on the CD36 signaling pathway. However, very little is known about the molecular mechanisms by which CD36 mediates carcinogenesis. In previous studies, CD36-mediated intracellular signaling could be initiated by the physical association of three members of the Src family of protein tyrosine kinases, namely Fyn, Lyn, and Yes [42-44]. In addition, some studies have reported that the interaction of exogenous lipids and CD36 can induce the phosphorylation of the non-receptor tyrosine kinase Src, which is implicated in a variety of cellular processes that are linked to cancer malignancy, such as cell proliferation, invasion, migration and survival. In the present study, both olive oil and OA induced a marked increase in Src phosphorylation, subsequently activating the ERK1/2 pathway in a CD36-dependent manner. Inhibition

of Src activity with SU6656 weakened the OA tumor-promoting phenotype. Our results suggest a key role of the Src/ERK1/2 pathway in the OA-mediated effects. However, how CD36 activates the Src tyrosine kinase is still not clear. According to previous reports, CD36 may not be directly associated with Src but may be indirectly mediated by the activation of other members of the Src family (Fyn, Lyn). However, further studies are required to elucidate this mechanism.

In conclusion, our study suggests that OA-induced CD36, by activating Src/ERK signaling pathway, could be a critical step in the development and progression of CC; thus, CD36 may be a novel target for cancer therapy.

Acknowledgments and funding

This research was supported by the National Natural Science Foundation of China (81570517, 31571210 and Key Program, No. 81390354), the Chongqing Research Program of Basic Research and Frontier Technology (No. cstc2015jcyjBX0044), and the Science and Technology Research Program of Chongqing Municipal Education Commission (NO. KJ1702029).

Conflict of interest

The authors have no conflicts of interest to disclose.

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ACCEPTED MANUSCRIPT

Figure legend:**Figure1. High olive oil diet feeding aggravates tumor growth and metastasis in a**

CC xenograft model. Nude mice fed with a normal diet or a high olive oil diet were inoculated subcutaneously with 5×10^6 HeLa cells into the left flank (n=5). (A) Growth curves of the tumors in each group monitored for 40-day period. Tumor diameter was measured every 3 day with a vernier calliper and the volume of the tumors was calculated using a standard formula of $\text{length} \times \text{width} \times \text{width} \times 0.5$. (B) Photographs of subcutaneous tumors after excision. (C) Final tumor weights in each group. In another experiment, HeLa cells (1×10^6 cells) were injected into the nude mice via tail vein to establish an experimental metastatic model (n=10-11). (D) Representative images of the metastasized livers in each group after 40 days. The incidence of liver metastasis was 1/11 in the normal diet group, and was 4/10 in the high olive oil diet group. The arrows indicate the metastatic nodules and the gross counts of the nodules is presented on the right. (E) Representative images of HE staining of the metastasized liver sections. Tumor areas were measured with Image J software and were shown on the right. * $p < 0.05$ compared with the normal diet group.

Figure2. Effects of OA supplement on cell proliferation, migration and invasion.

HeLa cells were treated with or without OA at different concentrations for the indicated times. (A) Dose-dependent effect of OA (0-100 μ M, 24h) on cell viability (n=6). (B) Dose dependent effect of OA (0-50 μ M) on cell proliferation at 48h (n=6). (C) Proliferation of cells treated with 5 μ M OA (n=6). (D) Cell cycle analysis was performed after OA (5 μ M) treatment for 48h using flow cytometry (n=5). (E) Colony-formation assay. Cells treated with or without OA (5 μ M) were cultured for 2 weeks, after that the cells were stained with crystal violet, and then the colony numbers were quantified (n=5). Migration (F) and invasion (G) assay. Migration and

invasion assay were performed by transferring HeLa cells to serum free media in the absence or presence of OA (2.5 μ M, 5 μ M) into inserts with 8 μ m pore size containing membranes coated with Matrigel or not. Migration and invasion times were 12h and 48h, respectively. Cell number refer to average number $\pm$ SEM per field counted at 200 $\times$ magnification (n=6). * p <0.05 compared with controls.

Figure3. CD36 expression in xenograft tumors and tissues of patient with cervical cancer. (A) IHC staining for PCNA and CD36 in the xenograft tumors from mice fed with normal diet or olive oil diet. Representative images are shown at 400 $\times$ magnification. The relative mRNA (B) and protein (C) expression of CD36 in the tumors from mice fed with normal diet or olive oil diet (n=5). The relative mRNA (D) and protein (E) expression of CD36 in Hela cells treated with or without OA (10 μ M) for 24h (n=5). β -actin served as an internal control. * p <0.05 compared with controls. (F-H) The relative mRNA expression of *CD36* in patients with CC at different tumor stages. The clinical data on cervical cancer were downloaded from the Cancer Genome Atlas (TCGA) pan-cancer datasets (<https://cancergenome.nih.gov/>). The comparison of *CD36* expression among different clinical stages (American Joint Committee on Cancer Staging System) was analyzed among 291 CC patients. * p <0.05 compared with the lowest tumor stage. (I) Kaplan-Meier survival curves showing overall survival of CC patients with high or low *CD36* expression. The prognostic significance between *CD36* low and high expression groups (with a threshold value of FPKM>0.9 or $\leq$ 0.9) was evaluated by the Kaplan-Meier survival method with Log rank test.

Figure4. CD36 overexpression promotes cell growth in vivo and in vitro. CD36 overexpression (CD36OE) stable HeLa cell line was constructed by transfection of a

recombinant lentivirus (Ubi-MCS-3FLAG-SV40-puromycin) containing CD36 cDNA or an empty vector as control (NC). Cell proliferation (A), cell cycle (B) and cell migration of the CD36 OE cells or control cells. Nude mice were inoculated subcutaneously with 5×10^6 CD36OE cells or control cells into the left flank (n=5). (D) Tumor volumes in each group were measured for 40-day period. (E) Photographs of subcutaneous tumors from each group are shown. (F) Tumor weights in each group. (G, H) IHC analysis of PCNA, CD36, Vimentin, and E-cadherin in tumors from each group. Representative images are shown at 400×magnification. * $p < 0.05$ compared with controls.

Figure5. Suppression of CD36 inhibits OA-induced cell growth and migration.

HeLa cells were pretreated with the CD36 inhibitor SSO for 15min, and then subjected to OA (10μM) loading. (A) Scratch-wound cell migration assays of cells treated with OA alone or in combination with SSO (n=5). (B) The histogram represents the area of migration in each group. Cell proliferation (C) and cell migration (D) of cells treated with OA alone or with SSO (n=5). CD36 knockdown (CD36 RNAi) cell line was established by transfecting CD36 shRNA lentiviral construct targeting sequence 5' -GGCTGTGTTTGGAGGTATTCT-3' or scrambled shRNA lentivirus as control. (E) Migration and invasion assay for the control and CD36 knockdown cells in the absence or presence of OA (10μM). Cell number refer to average number $\pm$ SEM per field counted at 200× magnification (n=5). (E) Proliferation of the control and CD36 knockdown cells in the absence or presence of OA (10μM) (n=6). * $p < 0.05$ compared with the control group; [#] $p < 0.05$ compared with the OA group.

Figure6. Src/ERK signaling pathway stimulation by OA modulate cell growth and migration. (A) Western blot analysis of Src, P-Src (tyr416), ERK1/2, P-ERK1/2 (Thr202/Tyr204), AKT, P-AKT (ser473), JNK, P-JNK (Thr183/Tyr185), AMPK and P-AMPK (Thr172) expression in tumors of mice fed with the normal diet or high olive oil diet. One of three representative experiments is shown. (B) Western blot analysis of Src and P- Src expression in CD36 OE cells or xenograft tumors, as well as the Src and ERK1/2 signal pathway in OA-treated cells and CD36 knockdown cells. One of three representative experiments is shown. (C) The histogram represents the densitometric scans for protein bands from A and B. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the NC+OA group. (D) Cell migration and invasion assay. HeLa cells were transferred to serum free media containing OA (10 μ M) alone or with Src inhibitor SU6656 (0.5 μ M) into inserts with 8 μ m pore size containing membranes coated with Matrigel or not (n=6). Cell number refer to average number $\pm$ SEM per field counted at 200 $\times$ magnification. (E) Proliferation of HeLa cells treated with OA alone or with SU6656 (n=6). * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the OA group. (F) Effects of CD36 knockdown on the proliferation and migration of HeLa cells treated with SU6656 (n=5).

Supplemental Figure 1

(A) Immunofluorescence of CD36 expression in the control and OA-treated cells. Representative images are shown at 630 $\times$ magnification. CD36 OE stable HeLa cell line was constructed by transfection of a recombinant lentivirus containing CD36 cDNA or an empty vector as control (NC). (B) Western blot analysis of CD36 expression in NC and CD36 OE cells. One of three representative experiments is shown. Nude mice were inoculated subcutaneously with NC cells or CD36OE cells.

(C) Western blot analysis of CD36 expression in NC and CD36 OE xenograft tumors. One of two representative experiments is shown. (D) CD36 knockdown cell line was established and confirmed by western blot. One of two representative experiments is shown. (E) Proliferation of the control and CD36 knockdown cells in the absence or presence of OA (5 μ M) (n=5). * p <0.05 compared with the control group; # p <0.05 compared with the OA group. (F) The effects of SU6656 on the phosphorylation of Src and ERK1/2.

Figure1

Figure2

Figure3

Figure4

Figure5

Figure6

Highlights:

1. Dietary oleic acid promotes the tumorigenesis of cervical cancer (CC) in vivo.
2. Oleic acid stimulates HeLa cell proliferation, migration, and invasion in vitro.
3. The fatty acid receptor CD36 plays a key role in oleic acid induced tumor-enhancing effects.
4. High CD36 expression induced by oleic acid may initiates intracellular signaling through Src tyrosine kinase to promote CC tumorigenesis and development.