Lauric acid can improve the sensitization of Cetuximab in KRAS/BRAF mutated colorectal cancer cells by retrievable microRNA-378 expression

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Abstract. EGFR-inhibitor (Cetuximab) is one of the main targeted drugs used for metastatic colorectal carcinoma (CRC). The benefit from Cetuximab appears to be limited to a subtype of patients, not for the patients with tumors harboring mutated BRAF or KRAS genes; unfortunately, it accounts for ~40-50% of CRC cases. Previous studies have connected higher expression levels of miR-378 to be commonly presented in patients without BRAF or KRAS mutants than in mutated CRCs. The microRNA-378 (miR-378) is coexpressed with PGC-1β and can be easily induced by fatty acid, for example lauric acid. Therefore, we hypothesized that elevation of miR-378 expression in mutated CRCs may stimulate the cell response to Cetuximab. Herein, seven CRC cell lines with confirmed mutation status were involved in two parallel experiments; directly *in vitro* transfected miR-378 mimics, and using lauric acid to indirectly induce the level of miR-378 in cells. After the increase of miR-378 in cells by either direct or indirect approaches, sensitivity to Cetuximab was restored in all BRAF mutants (p-value <0.0001-0.0003), and half of KRAS mutants CRC (p-value 0.039-0.007). Further evidence was gained by decreasing expression of MEK and ERK2 proteins after transfection with miR-378; it was similar to the indirect induction by lauric acid approach. In conclusion, the present study demonstrated that lauric acid may efficiently induce miR-378 expression in CRC mutants, and both BRAF and a subtype of KRAS mutants presented significantly improved sensitivity to Cetuximab. Notably, BRAF mutants could even be inhibited in cell proliferation after elevated concentration of miR-378 in cells without combining with targeted therapy. This new approach may shed new light on BRAF or KRAS mutation in CRC patients for clinical trial, since lauric acid may easily be obtain from natural food, and it is supposed to be harmless to the cardiovascular system.

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Introduction

Metastatic colorectal cancers (mCRCs) are known to benefit from targeted therapy of epithelial growth factor receptor (EGFR) inhibitor, including Cetuximab and Panitumumab. However, Cetuximab is ineffective in patients harboring BRAF and KRAS mutations, which accounted for 40 and 10% of CRC patients, respectively (1-6). The phenomenon of mutually exclusivity between mutated BRAF and KRAS in CRCs in a same tumor have been particularly noted (7,8). The high correlation between BRAF and KRAS mutation status and ERK1/2 activation has been proven in many types of cancer included CRC. Nevertheless, once CRCs contain mutation of either BRAF or KRAS genes, Cetuximab cannot suppress the auto-signal transduction downstream of the signaling pathway, and could even trigger uncontrolled abnormal cell growth, proliferation and even metastases (4,9).

The miR-378 is a very short non-coding RNA, reported to possibly play a crucial role in mediating gene expression in CRC cells. It was considered to function as a tumor suppressor in inhibiting tumor growth and invasion; aberrant expression of miR-378 results in dysregulation of cell proliferation, increase the tumor size, as well as the capability of tumor cell invasion (10). Previous studies indicated that miR-378 could act as a new biomarker in CRC, due to higher expression level only observed in $_{wt}$CRC (the CRC without presenting BRAF or KRAS mutations) or normal of colonic tissues but not in BRAF or KRAS mutated CRC cells (11,12). Most recently, high expression of miR-378 can even suppress the cell proliferation and induces apoptosis by targeting BRAF, as observed by Wang *et al* (13). Although the correlation between miR-378 and MAPK signaling

pathway were revealed by substantial evidence, for example, Nagalingam *et al* emphasized Ras signaling could be one of the main targets of miR-378 in cardiac hypertrophy (14), however, the underlying mechanism of different subtypes of CRC, which correlated with sensitivity to the targeting therapy or regulation of miR-378 still remain unknown.

The precursor of miR-378 is derived from the first intron of peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β) gene, a cellular energy-related gene. Expression of PGC-1β is highly associated with saturated fatty acid intake (Fig. 1) (15-19). We therefore postulated that once CRC cells were induced by lauric acid, the saturated fatty acid with a 12-carbon atom chain, triggers the transcriptional activity of PGC-1β gene to accumulate messenger RNA (mRNA) expression, consequently resulting in increasing level of coexpressed products of miR-378 in cells (Fig. 1); once elevated the expression level of miR-378 in CRC mutants to the level of $_{wt}$CRC cells or normal cells, it may automatically suppress cell growth or lead the mutated CRC cells restoring sensitivity to Cetuximab. In the present study, the elevation of miR-378 expression levels in BRAF or KRAS mutated CRC cells were firstly restored by an *in vitro* transfection method; then the cells were incubated in lauric acid growth medium, and their response to Cetuximab was further measured via cellular viability. In addition, we performed protein analysis to investigate the potential role of miR-378 associated with MAPK signaling pathway.

Materials and methods

Confirmation of BRAF and KRAS mutational status of the cell lines. Seven colon cancer cell lines were used, these were HCT-116, HCT-15, SW-480, SW-620, WiDr, HT-29 and Caco-2. The mutation statuses of both BRAF and KRAS of each cell line was confirmed by sequencing analysis, and further searched according to the COSMIC database (http://www.sanger.ac.uk/genetics /CGP/cosmic/) before the experiments. KRAS mutation with G13D substitution in exon 2 was confirmed in HCT-116 and HCT-15, SW480 and SW620 are KRAS mutated with G12V in exon 2. HT-29 and WiDr are BRAF mutated with V600E in exon 15. The mutation of BRAF and KRAS did not coexist in any of the cell line used. Caco-2 was confirmed as non-BRAF or non-KRAS mutation, labeled as $_{wt}$CRC, which is reported to be sensitive to Cetuximab according to clinical experience (20-23), was used as internal control when needed. The cells were cultured in RPMI or Dulbecco's modified Eagle's medium (DMEM) growth medium with 10-20% fetal bovine serum (FBS). Indeed, for the time of lauric acid treatment *in vitro*, we could only select the more aggressive cell growth, such as HCT116, SW480 and HT29, in order to further obtained proteins for detection and observed differences in cell viability.

Elevated level of miR-378 in cells by direct in vitro miR-378 transfection. Directly transfected miR-378 to all CRC cell lines were performed by seeding into a 6-well plate with $1x10^5$ cells/well and cultured overnight at 37˚C with 5% CO_2. miR-378 transfection complexes were then prepared by mixing with miR-378 mimic and HiPerFect Transfection Reagent (cat. no. 301704) (both from Qiagen, Valencia, CA, USA) under instructions of the manufacturer's protocol. To confirm successful miR-378 transfection, the commercially available positive control AllStars Hs Cell Death control siRNA (cat. no. 1027298), and Allstars negative control siRNA validated non-silencing siRNA (cat. no. 1027280) (both from Qiagen) were included in every batch of the experiments. Before and after transfection of miR-378, the expression level was detected by qRT-PCR, and the commercial available normal colon RNA extraction (BioChain, Newark, CA, USA) was used as baseline comparison.

Elevated level of miR-378 in cells by indirectly incubating cells in lauric acid medium. In order to indirectly stimulate cells to coexpress miR-378 from its host gene PGC-1β by feeding lauric acid (15-19), the three cell lines (HT29, HCT116 and SW480) with aggressive growth patterns were selected and cultured in a gradient lauric acid concentration (0.15, 0.3, 0.45 and 0.6 mM) of growth medium for 96 h. The metabolic rates of lauric acid in cells were then further measured before the RNA expression level of PGC-1β, and coexpressed miR-378 levels were investigated.

RNA extraction and quantification of miR-378. The expression level of miR-378 in cells was detected at original status cells, before and after miR-378 transfection *in vitro* or indirectly induced miR-378 by lauric acid feeding. Extraction of total RNA was performed using TRIzol (Invitrogen Inc., Carlsbad, CA, USA), cDNA of miR-378 was synthesized according to the TaqMan microRNA assay protocol (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan Universal Master Mix, and 20 times volume of miR-378 primer (TaqMan microRNA assay). The expression level of miR-378 was quantified by comparative CT method with an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction, the expression level was quantified as the relative quantitative (RQ) level, in which RNU-44 was used as an endogenous control. Normal colon total RNA extract (BioChain) was utilized as normal control in the experiments.

Optimal concentration of Cetuximab-resistant test. The optimal concentration of Cetuximab (Merck Serono) resistant test was determined by flow cytometry with Annexin V-FITC apoptosis detection test (Annexin V-FITC apoptosis detection kit II; BD Pharmingen™) after incubation of cells with Cetuximab for 24 h through a gradient concentration of 0, 20, 50, 100, 120 and 200 μg/ml. The final drug concentration of 100 μg/ml was determined to be the minimal concentration that provided an effective steady apoptotic effect on all cells.

Cell viability analysis. To determine the number of viable cells in culture, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was performed before and after miR-378 transfection, lauric acid and Cetuximab treatment. In brief, a total of $5x10^3$ cells were cultured in a 96-well plate/well; and then cultured with different concentration of

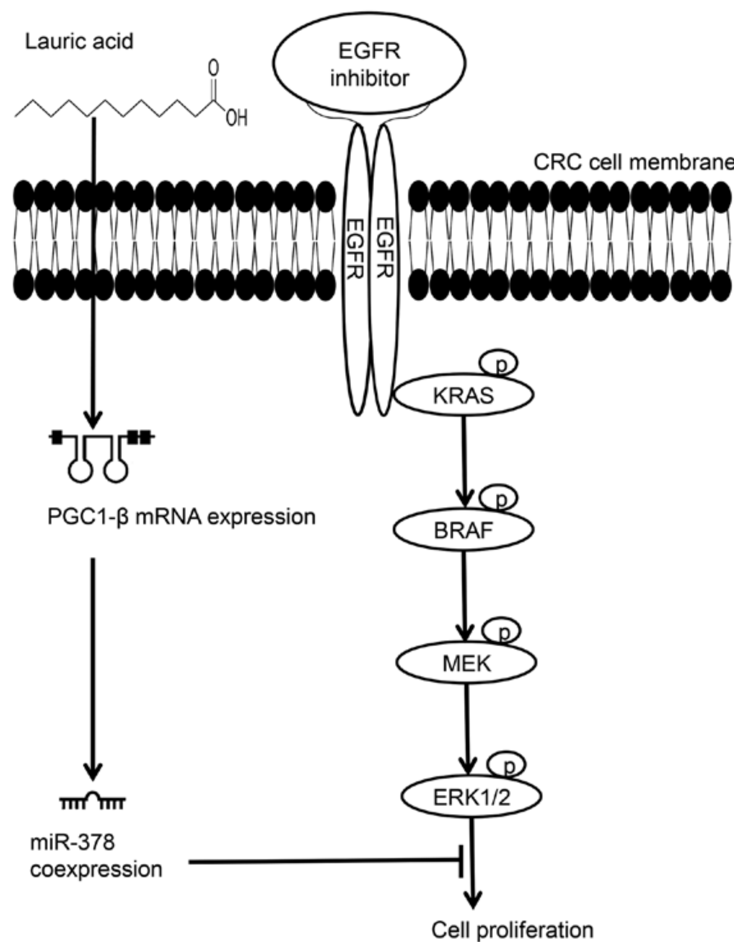

Figure 1. The sensitivity of anti-EGFR antibody inhibits the RAS/RAF/MAPK pathway leading to cell cycle progression. miR-378 is induced by lauric acid stimulated PGC1-β mRNA expression further blocking the ERK1/2 protein activity resulting in cell death.

lauric acid (0.15, 0.3, 0.45 and 0.6 mM) for 96 h. For the drug testing, 100 µg/ml Cetuximab was added and for another 72 h. Before detection of cell viability, 20 µl of CellTiter 96® AQueous One Solution Reagent was added and cultured at 37˚C in an incubator for 1 h, and the the absorbance at 490 nm was recorded with a 96-well plate reader.

Activation of MAPK pathway analysis for the transfected miR-378 cells. The bioinformatic prediction suggested that factors of the mitogen-activated protein kinase (MAPK) pathway are enriched among miR-378 targets. Therefore, western blotting was performed to analyze the MAPK pathway related proteins before and after transfection of miR-378 cells according to the standard protocol. In brief, cells were washed with phosphate-buffered saline (PBS) and then trypsinized (0.05% trypsin w/v with 0.02% EDTA). The pellets were lysed in buffer [50 mM Tris-HCl, 10 mM EDTA, 1% v/v Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF), 0.05 mM pepstatin A and 0.2 mM leupeptin], and after mixing with sample loading buffer (50 mM Tris-HCl, pH 6.8, 10% w/v sodium dodecyl sulfate, 10% v/v glycerol, 10% v/v 2-mercaptoethanol and 0.04% bromophenol blue) at a ratio of 4:1 were denatured at 95˚C for 5 min. Protein (30 µg) was then loaded into 10% SDS-polyacrylamide (SDS-PAGE) gels and subjected to electrophoresis (120 V, 75 min). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad; 40 min, 350 mA/gel). The blots were incubated and slightly shacken in 5% non-fat milk/TBS-Tween-20 blocking buffer for 1 h, followed by overnight incubation at 4˚C with different ratio dilution of the primary antibodies: 1:500 dilution of anti-KRAS (mouse monoclonal, ab55391), 1:2,500 dilution of anti-BRAF (rabbit monoclonal, ab33899), 1:2,000 dilution of anti-MEK (mouse monoclonal, ab69502), 1:2,500 dilution of anti-ERK (mouse monoclonal, ab36991) (Abcam, Cambridge, MA, USA). After washing with TBS, the blots were incubated at room temperature for 1 h with the secondary antibody. Protein detection and quantification by densitometric analysis were performed after normalization with β-actin (mouse monoclonal, NB600-501; Novus Biologicals, Littleton, CO, USA).

Enzyme-linked immunosorbent assay using ERK 1/2 protein in the lauric acid fed cells. Amounts of proteins derived from 0.45 mM lauric acid fed CRC cells (HT29, HCT116 and SW480), with or without 0.6 µM Cetuximab, were analyzed using ERK 1/2 (Total) InstantOne™ enzyme-linked immunosorbent assay (ELISA) according to the instructions (Affymetrix, eBioscience®).

Statistical analysis. In order to eliminate any background variation among the experiments, each original CRC cell line was used as a reference group to normalize the following five

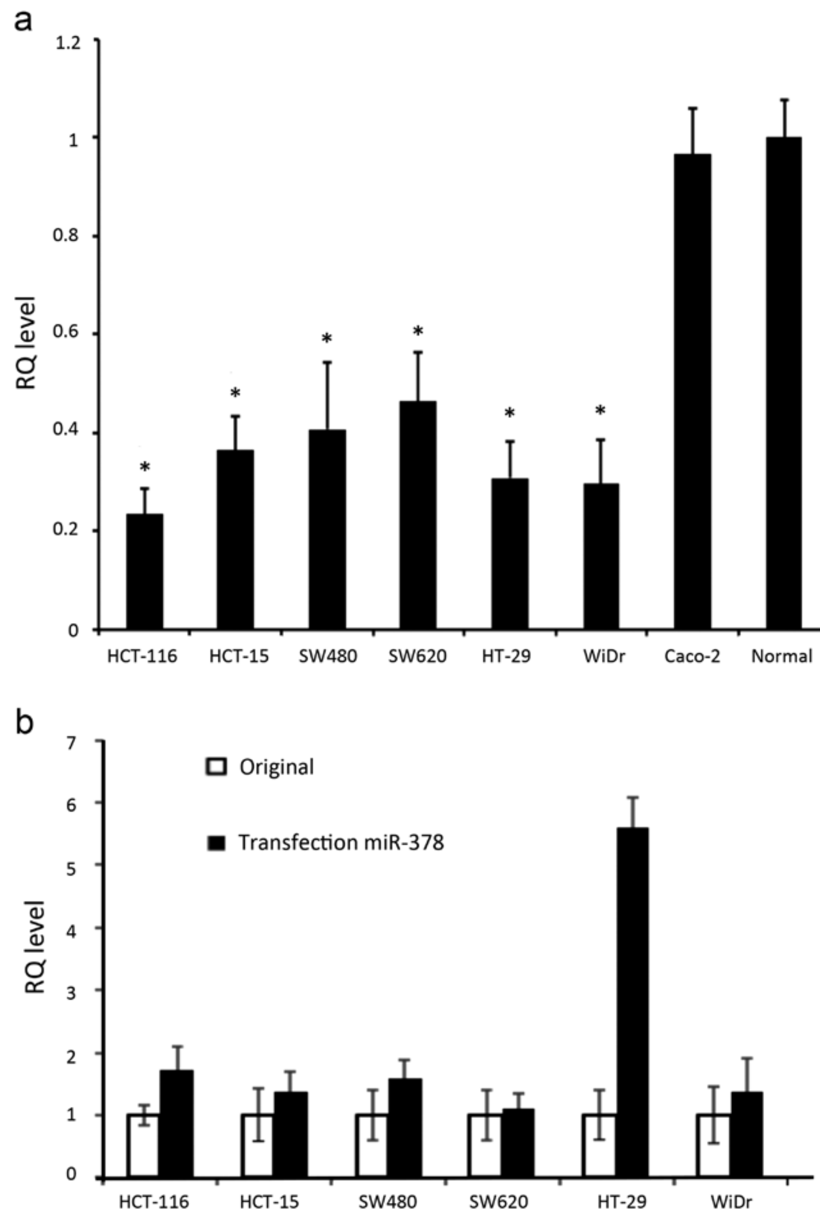

Figure 2. Relative expression of miR-378 in each CRC cell line compared to $_{wt}$CRC cells and normal control. (a) Downregulation of miR-378 was shown in both BRAF and KRAS mutants; (b) relative expression of miR-378 of CRC cells before and after miR-378 transfection *in vitro*. All original cell lines were normalized to RQ=1, *p<0.0001. RQ level, relative quantitative level.

groups: cells treated with Cetuximab only, cells transfected with miR-378 only, miR-378 transfected cells combined with Cetuximab treatment, lauric acid incubated only, and lauric acid incubated combined with Cetuximab treatment. Analysis were performed with SPSS 15.0 statistics software (SPSS, Inc., Chicago, IL, USA). The difference between miR-378 expression in colon cancer cell lines and normal colon extract was analyzed by single variant post hoc test. Paired comparisons of percentage cell viability analysis of original cells, miR-378 transfected cells with, and without Cetuximab treatment were analyzed with Student's t-test. A p-value of ≤0.05 was considered to indicate a statistically significant result.

Results

Significantly elevated expression level of miR-378 in cells by directly transfected miR-378 or indirectly induced by lauric acid. The expression levels of miR-378 in all original mutants were significantly lower than the normal control and Caco-2 (p<0.0001) (Fig. 2a). We succeeded in transfecting miR-378 to all CRC cell lines, confirmed by the parallel experiments of AllStars Hs Cell Death Control siRNA. All CRC cells significantly increased their expression levels of miR-378 after *in vitro* transfection, in which HT-29 showed the highest efficiency with 5.6-fold and 1.1-fold elevation for SW620 (Fig. 2b). Similarly, three selected aggressive growth pattern cells (HT29, HCT116 and SW480) after incubated in lauric acid culture medium for 24-72 h, also presented increased mRNA level of PGC-1β and also coexpressed miR-378 in cells (Fig. 3a-c).

All cells restored sensitivity to Cetuximab after elevation of the level miR-378 in cells. The cell viability was significantly decreased after treatment with Cetuximab both in

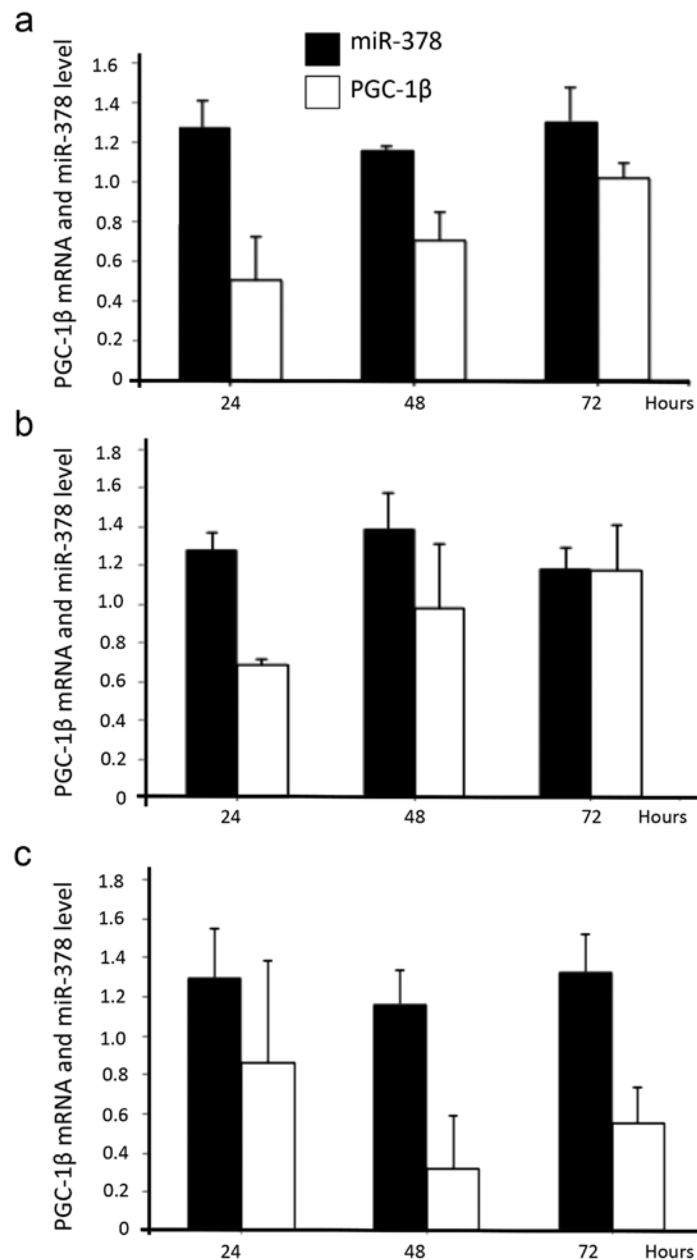

Figure 3. Three selected cell lines were incubated in lauric acid for 24, 48 and 72 h to stimulate the PGC1β mRNA expression, and coexpressed miR-378. Black bars represent expression level of miR-378 in cells; white bars represent expression level of mRNA PGC-1β in cells. (a) HT29; (b) HCT116; (c) SW480.

miR-378 transfected mutants and lauric acid incubated cells (Figs. 4a and 5; Table I). Notably, the cell viability of miR-378 transfected KRAS mutants HCT116 and SW620, were significantly decreased after treatment with Cetuximab when compared to the cells treated with Cetuximab only (p=0.007 and 0.39, respectively); a similar response also occurred in the BRAF mutants, HT29 (p<0.0001) and WiDr (p=0.0003). Although there was no statistical significance in the HCT15 and SW480 cells, increasing cell viability should be not ignored when miR-378 transfected before treatment with Cetuximab (Fig. 4a; Table I).

MEK and ERK1/2 protein detection after increasing miR-378 expression level in cells. To investigate the MAPK pathway proteins, western blot analysis was performed. Despite KRAS protein, lower protein products of BRAF were observed in either KRAS or BRAF mutants. Similar results were found in EKR2 protein. However, lower MEK protein was observed in all mutants, except in two KRAS mutants (Fig. 6). Parallel experiments of ERK1/2 proteins were performed by ELISA for the lauric acid induced coexpressed miR-378 in three selected aggressive growth CRC cells (HT29, HCT116 and SW480). Lower protein expression of ERK1/2 were observed after CRC cells were incubated in lauric acid medium and compared to the original cells or Cetuximab-treated only cells (Fig. 4b). Apparently, decreased percentage of cell survival followed by increasing concentration of lauric acid that was absorbed by cells, and even regardless of the mutation types of the cells, all the cells incubated in 0.6 mM lauric acid growth

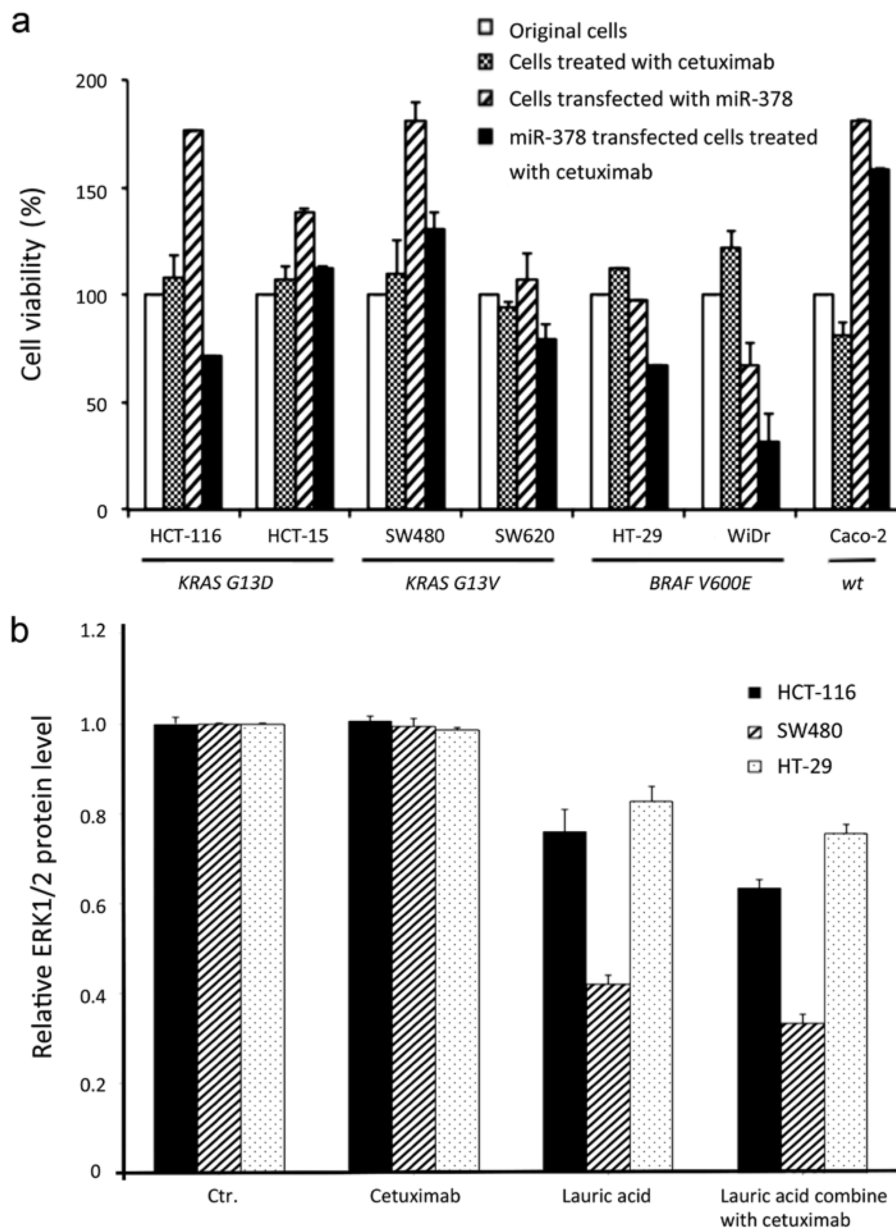

Figure 4. (a) Percentage changes of cell viability after treatment with 0.6 μM Cetuximab, after transfection with miR-378, transfected miR-378 cells treated with Cetuximab with respect to the original untreated cells. All original cell lines were normalized to 100%; (b) comparison of ERK1/2 protein expression level among the groups, included control (Ctr) cells, treated with 0.6 μM Cetuximab=, incubated in 0.45 mM lauric acid, or combined Cetuximab and lauric acid. Each control group was normalized as basal line and compare to the other groups.

medium presented significant sensitivity at all dosages of Cetuximab tested (p<0.001-0.005) (Fig. 5a-c).

Discussion

To improve the benefit to CRC patients with BRAF or KRAS mutations in target drug-Cetuximab therapy, we restored the expression level of miR-378 in BRAF or KRAS mutated CRC cells in two ways: i) directly *in vitro* transfected miR-378 into cells (Fig. 2b); ii) induced the transcriptional activity of PGC1-β gene to produce mRNA by incubating cells in lauric acid growth medium, and consequently indirectly stimulated coexpression of miR-378 in cells (Fig. 3). We demonstrated lower cell viabilities were strongly linked to higher expression level of miR-378 present in CRC mutated cells, and it further significantly improved the sensitivity of the mutants to Cetuximab (Fig. 4a and Table I). To uncover the possible underlying mechanism between miR-378 and Cetuximab, the association of the proteins with MAPK pathway was investigated. Generally, our findings demonstrated coincident results from parallel experiments both directly *in vitro* transfected miR-378 into CRC cells and indirectly induced miR-378 by lauric acid (Figs. 4b and 6). Hence, lower level products of MEK, ERK proteins and decreasing cell viability provided evidence to indicate miR-378 inhibited cell proliferations and triggered cell apoptosis (Weng *et al*, unpublished data), particularly in BRAF mutants (Fig. 4); the phenomena led us to evaluate and address the characteristics of miR-378 in modulating certain molecules in MAPK signaling pathway in CRCs.

Table I. Statistical significance of changes in cell viability was analyzed by comparison between the status of two different cells, included untreated original cells, cells treated with Cetuximab, cells transfected with miR-378, miR-378 transfected cells treated with Cetuximab.

Mutation type/cell line	Treated with Cetuximab	Transfected with miR-378	miR-378 transfected cell treated with Cetuximab
KRAS mutation			
HCT-116			
Untreated cell	0.294	**0.0003**	**0.0002**
Transfected with miR-378			**0.0001**
Treated with Cetuximab			**0.007**
HCT-15			
Untreated cell	0.084	**<0.0001**	**<0.0001**
Transfected with miR-378			**<0.0001**
Treated with Cetuximab			0.081
SW480			
Untreated cell	0.508	**0.008**	**0.042**
Transfected with miR-378			**0.008**
Treated with Cetuximab			0.160
SW620			
Untreated cell	**0.01**	0.334	**0.005**
Transfected with miR-378			**0.001**
Treated with Cetuximab			**0.039**
BRAF mutation			
HT-29			
Untreated cell	**0.01**	**0.03**	**0.001**
Transfected with miR-378			**<0.0001**
Treated with Cetuximab			**<0.0001**
WIDR			
Untreated cell	0.074	0.059	**0.025**
Transfected with miR-378			**0.028**
Treated with Cetuximab			**0.0003**
Wild-type			
Caco-2			
Untreated cell	**0.015**	**0.0004**	**0.0004**
Transfected with miR-378			**0.012**
Treated with Cetuximab			**<0.0001**

Bold numbers indicate significance with p-value ≤0.05.

The linkage of miR-378 bound MAPK signaling pathway associate with the targeting drug-Cetuximab and CRC the mutants interaction was hypothesized in the present study. Thus, we firstly confirmed the status of BRAF and KRAS mutation of all CRC cells, then tested insensitive to Cetuximab. The results showed the mutants did not respond to the drug, except $_{wt}$CRC (Caco-2) (Fig. 4a). We then further observed the original basal miR-378 expression levels of all BRAF and KRAS mutants, both were significantly lower than normal control cells and $_{wt}$CRC cells (Fig. 2a and Table I). A similar finding of lower expression of miR-378 in the BRAF mutated CRC cells were recently demonstrated by Wang *et al* (13). Our results supported by previous reports that pinpointed the expression of miR-378 in mutated CRC tumors differ from normal tissues or $_{wt}$CRCs (11). Obviously, after the cells were transfected with miR-378 *in vitro* to mutants, all BRAF and KRAS mutated cells reversed the drug sensitivity to Cetuximab; simultaneously presented decreasing cell viabilities ($p<0.028$-0.0001) (Fig. 2a and Table I). Though all the cells presented significant response to Cetuximab after transfection of miR-378 into cells; only half of the KRAS mutants (HCT116 and SW620) showed 'real' significant re-sensitization to Cetuximab when compared the the cell viability of Cetuximab treated-only cells (Fig. 4a and Table I). We

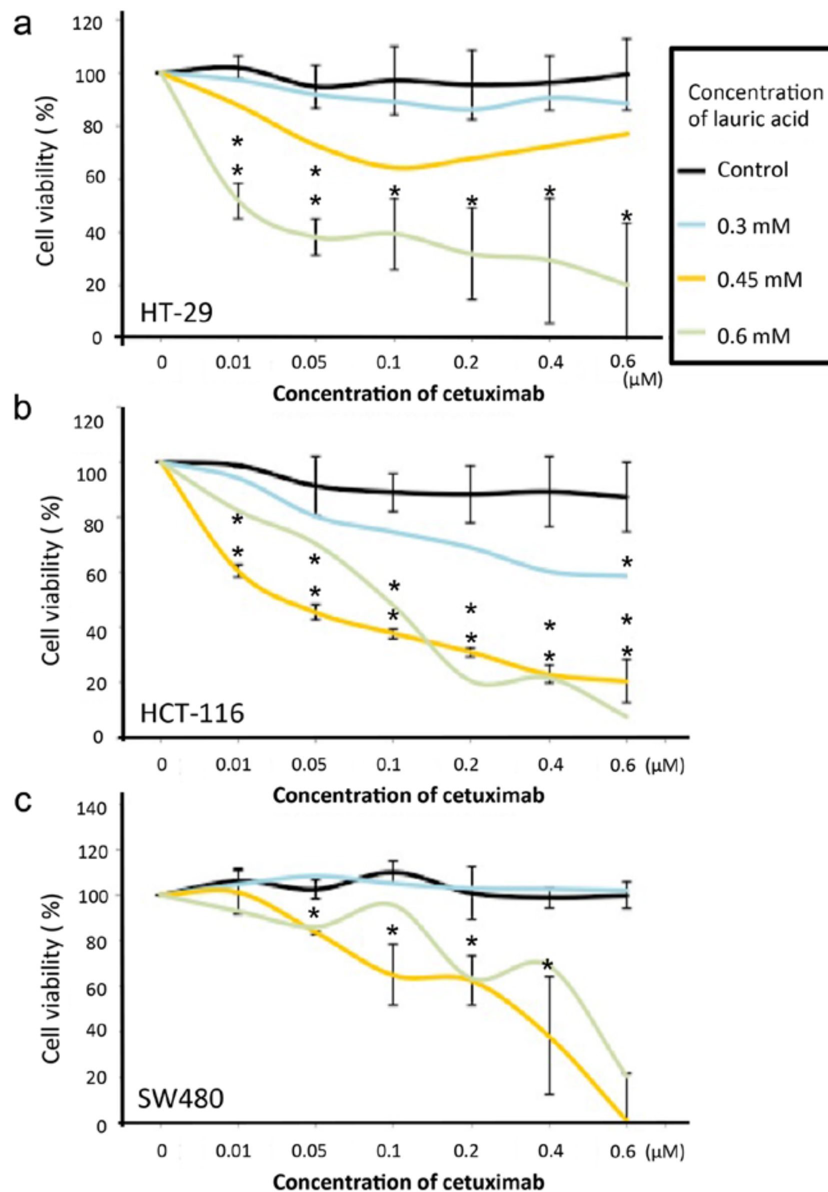

Figure 5. Three selected cells cultured in grading concentration of lauric acid (0-0.6 mM), and combined treated with different dosages of Cetuximab (0-0.6 μM). (a) HT29; (b) HCT116; (c) SW480. The mean values ± SD were analyzed; $^{*}p<0.05$, $^{**}p<0.01$.

assumed that possible subtypes of KRAS mutant CRCs may exist with unknown molecular characteristics. This phenomenon strongly suggested miR-378 plays a crucial role in modulating mutated CRC cells to respond to Cetuximab. Apparently, stable high expression level of miR-378 is required for CRC cells response to Cetuximab, irrespective of their BRAF or KRAS status (Fig. 4a, Table I) (24).

We noted a contradictory effect on the cell survival between BRAF and KRAS mutated CRC cells after performing miR-378 transfection *in vitro*. The cell growth inhibition was observed in the BRAF mutants; contrarily, increasing cell growth occurred in all KRAS mutants as well as in Caco-2 (Fig. 4a). Similar findings have been discussed in the study of Mosakhani *et al*, indicating the roles of miR-378 may act as an effector to stimulate cell proliferation, although the reason remain still unknown (11). It is also known that miR-378 acts as a Myc target, and modulates the c-Myc/TOB2/cyclin D1 transformation signaling pathway. Later on miR-378 is involved in activating Ras and EGFR signaling transduction, and acts as a downstream effector of the oncogenic EGFR-Ras-ERK pathway (25). Once a mutation occur in one of the genes it could lead to CRC, although they are considered as independent prognostic factors (26). The contrary evidence was provided by recent finding that emphasized miR-378-5p (5-cuccugacuccagguccugugu-26, the sequence same as the present study) suppresses cell proliferation and induces apoptosis in CRCs by targeting BRAF (13), similar data from the present study also show obviously lower BRAF protein expression in the BRAF-mutated CRCs after transfection of miR-378 *in vitro* (Fig. 6). Therefore, it may be the reason why transfection of miR-378 into cells trigger cell proliferation in KRAS mutants, but not in BRAF mutants, *i.e.* due to the molecular alterations and miR-378 modulating in MAPK pathway (Fig. 4a and Table I). Indeed, increasing evidence supports that miR-378 is widely accepted as having diverse roles in carcinogenesis, and

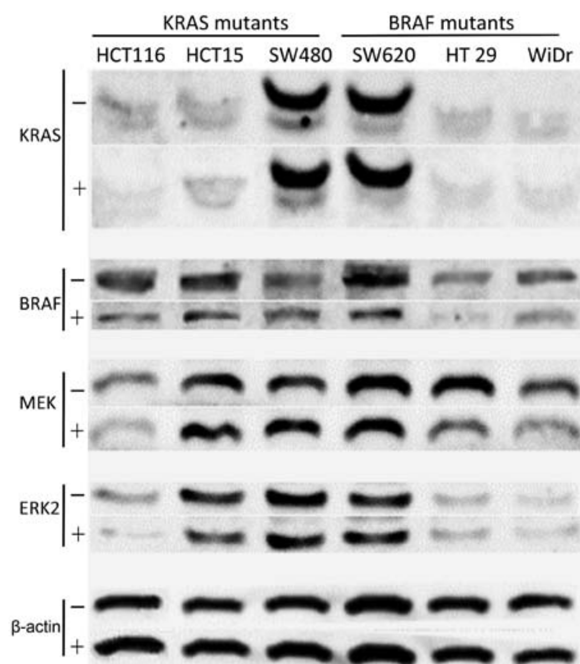

Figure 6. Western blot analysis of the proteins correlated with MAPK pathway applied on KRAS and BRAF mutants. '-' represents the cells derived before miR-378 transfection; '+' represents the cells derived after miR-378 transfection.

involved in the regulation of tumor either in proliferation or apoptosis; and may act as an oncogene or tumor suppressor depending on the targeting of mRNAs or tumors (27-32). Accordingly, we speculated that miR-378 may behave as an effector to manipulate several proteins that involve in the MAPK signaling pathway, and/or block the signaling transduction to preclude the possibility of promotional tumorigenesis. This phenomenon provides information suggesting clinicians may need to make the treatment distinct between BRAF-and KRAS-mutation patients due to their underlying molecular differences. Despite increased cell growth of the KRAS mutants induced by increasing miR-378 amount in cells, it was still overcome by an even higher efficiency in restoring the sensitivity to Cetuximab of miR-378 transfected KRAS mutants (Fig. 4a and Table I).

Based on above findings, we tried to find possible natural resources for clinically practical usage. The precursor of miR-378 coexpressed with PGC-1β mRNA, and was easily induced by saturated oil as previously documented (23,29). The miR-378 is located in the first intron of the PGC-1β gene, and coordinately expressed with PGC1 (24). The expression of PGC-1β is highly inducible in response to the dietary intake of saturated fats *in vivo* (Fig. 1) (19). Herewith, we elevated the expression level of miR-378 in CRC cells using saturated lauric fatty acid, which contains a 12-carbon atom medium chain and was selected to apply in a series of experiments. Lauric acid is a common fatty acid easily be found in milk, and much more in many vegetable fats, particularly in coconut and palm kernel oils (22). Although it is a saturated fatty acid, it was characterized as having a more favorable effect on total high-density lipoprotein (HDL) cholesterol than any other fatty acid, either saturated or unsaturated (33). Therefore, it has been generally used, and believed it could be applied in medical treatment, such as viral infections, yeast infections and anti-bacteria (34). More evidence associated to CRC was mentioned by Fauser *et al*, who emphasized induction of apoptosis by the lauric acid in the CRC cells were potentially resulted from oxidative stress (35). In the present study, in spite of *in vitro* transfected miR-378, we used lauric acid to stimulate the transcriptional activity of the PGC1-β gene and due to the strict experimental time schedule for lauric acid incubation and harvest, we therefore only selected three CRC cell lines with aggressive growth curve (HT29, HCT116 and SW480) for testing. After grading scale of lauric acid dosage supplied to cells, the elevation of the miR-378 levels in cells were followed by increase in expression of mRNA of PGC-1β; the inhibition of protein ERK1/2 was present in all cells, consequently leading to decrease of cell proliferation (Figs. 3 and 4b). Similar data supported by Fauser *et al* also proved successfully induced apoptosis of CRC cells by lauric acid treated *in vitro* (35). Our findings imply a possible strategy that should be kept in mind, the mutated CRC cells may simply apply with elevated amount of miR-378 in cells without combining with targeting drug treatment and still efficiently limited the tumor growth (Fig. 4b). Nevertheless, after the lauric acid induced either BRAF or KRAS, mutants tend to present similar results, where decreasing cell viability was followed by increasing the concentration of Cetuximab treatment (Fig. 5). Moreover, in all mutants lower ERK1/2 protein expression was observed either in lauric acid induced growth medium-only or lauric acid combined with Cetuximab treatment (Fig. 4b). These findings provide clinicians a straight, practical and not harmful usage to remedy current difficulties in applying targeted therapy in CRC patients with BRAF or KRAS mutations.

To uncover the mechanism of miR-378 activity correlated with the protein MAPK pathway. KRAS, BRAF, MEK and ERK1/2 proteins were included, and the protein quantities were measured before and after miR-378 transfection *in vitro* or lauric acid was induced, and then compared the cell viability between with or without Cetuximab treatment (Figs. 4b and 6). Variant levels of protein inhibition were observed in both MEK and ERK proteins when directly transfected with miR-378 in CRC cells, particularly the BRAF mutants (Fig. 6). Moreover, the consistent results of lower ERK1/2 protein expression were also demonstrated in lauric acid-induced cells either in BRAF- or KRAS-mutants (Fig. 4b).

In conclusion, the present study revealed a novel molecular mechanism that tightens the bonds between miR-378 and MAPK pathway, and the association with the efficiency of Cetuximab treatment. The fact that lauric acid induced miR-378 expression in cells affects the sensitivity of the mutated CRC cells to Cetuximab. Hence, high expression level of miR-378 may serve as a treatment modality for CRCs, particularly in BRAF mutation CRC; combination with Cetuximab may even impact the CRC patient treatments. Hopefully, our findings could provide a useful strategy to promote the use of Cetuximab on BRAF or KRAS mutated CRCs.

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References

1. Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomasic G, Penna C, Ducreux M, *et al*: *KRAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66: 3992-3995, 2006.
2. Di Fiore F, Blanchard F, Charbonnier F, Le Pessot F, Lamy A, Galais MP, Bastit L, Killian A, Sesboüé R, Tuech JJ, *et al*: Clinical relevance of *KRAS* mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 96: 1166-1169, 2007.
3. Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, Wong TW, Huang X, Takimoto CH, Godwin AK, *et al*: Expression of epiregulin and amphiregulin and *K-ras* mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 25: 3230-3237, 2007.
4. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, *et al*: Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634, 2008.
5. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, Siena S and Bardelli A: Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 67: 2643-2648, 2007.
6. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, *et al*: Wild-type *BRAF* is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 26: 5705-5712, 2008.
7. Gibbs JBSI, Sigal IS, Poe M and Scolnick EM: Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc Natl Acad Sci USA 81: 5704-5708, 1984.
8. Garnett MJ and Marais R: Guilty as charged: B-RAF is a human oncogene. Cancer Cell 6: 313-319, 2004.
9. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, *et al*: *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765, 2008.
10. Zhang GJ, Zhou H, Xiao HX, Li Y and Zhou T: MiR-378 is an independent prognostic factor and inhibits cell growth and invasion in colorectal cancer. BMC Cancer 14: 109-118, 2014.
11. Mosakhani N, Sarhadi VK, Borze I, Karjalainen-Lindsberg ML, Sundström J, Ristamäki R, Osterlund P and Knuutila S: MicroRNA profiling differentiates colorectal cancer according to *KRAS* status. Genes Chromosomes Cancer 51: 1-9, 2012.
12. Faltejskova P, Svoboda M, Srutova K, Mlcochova J, Besse A, Nekvindova J, Radova L, Fabian P, Slaba K, Kiss I, *et al*: Identification and functional screening of microRNAs highly deregulated in colorectal cancer. J Cell Mol Med 16: 2655-2666, 2012.
13. Wang Z, Ma B, Ji X, Deng Y, Zhang T, Zhang X, Gao H, Sun H, Wu H, Chen X, *et al*: MicroRNA-378-5p suppresses cell proliferation and induces apoptosis in colorectal cancer cells by targeting BRAF. Cancer Cell Int 15: 40, 2015.
14. Nagalingam RS, Sundaresan NR, Gupta MP, Geenen DL, Solaro RJ and Gupta M: A cardiac-enriched microRNA, miR-378, blocks cardiac hypertrophy by targeting Ras signaling. J Biol Chem 288: 11216-11232, 2013.
15. Walczak R and Tontonoz P: Setting fat on fire. Nat Med 9: 1348-1349, 2003.
16. Crunkhorn S, Dearie F, Mantzoros C, Gami H, da Silva WS, Espinoza D, Faucette R, Barry K, Bianco AC and Patti ME: Peroxisome proliferator activator receptor gamma coactivator-1 expression is reduced in obesity: Potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. J Biol Chem 282: 15439-15450, 2007.
17. Gerin I, Bommer GT, McCoin CS, Sousa KM, Krishnan V and MacDougald OA: Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis. Am J Physiol Endocrinol Metab 299: 198-206, 2010.
18. Carrer M, Liu N, Grueter CE, Williams AH, Frisard MI, Hulver MW, Bassel-Duby R and Olson EN: Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378. Proc Natl Acad Sci USA 109: 15330-15335, 2012.
19. Lin J, Yang R, Tarr PT, Wu PH, Handschin C, Li S, Yang W, Pei L, Uldry M, Tontonoz P, *et al*: Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. Cell 120: 261-273, 2005.
20. Wilson PM and Lenz HJ: Integrating biomarkers into clinical decision making for colorectal cancer. Clin Colorectal Cancer 9 (Suppl 1): S16-S27, 2010.
21. Cushman-Vokoun AM, Stover DG, Zhao Z, Koehler EA, Berlin JD and Vnencak-Jones CL: Clinical utility of KRAS and BRAF mutations in a cohort of patients with colorectal neoplasms submitted for microsatellite instability testing. Clin Colorectal Cancer 12: 168-178, 2013.
22. Dollah S, Abdulkarim SM, Ahmad SH, Khoramnia A and Ghazali HM: Physicochemical properties and potential food applications of *Moringa oleifera* seed oil blended with other vegetable oils. J Oleo Sci 63: 811-822, 2014.
23. Nelson VM and Benson AB III: Status of targeted therapies in the adjuvant treatment of colon cancer. J Gastrointest Oncol 4: 245-252, 2013.
24. Yu J, Kong X, Liu J, Lv Y, Sheng Y, Lv S, Di W, Wang C, Zhang F and Ding G: Expression profiling of PPARγ-regulated microRNAs in human subcutaneous and visceral adipogenesis in both genders. Endocrinology 155: 2155-2165, 2014.
25. Feng M, Li Z, Aau M, Wong CH, Yang X and Yu Q: Myc/miR-378/TOB2/cyclin D1 functional module regulates oncogenic transformation. Oncogene 30: 2242-2251, 2011.
26. Kruszewski W, Kowara R, Rzepko R, Warezak C, Zieliński J, Gryglewski G, Kopacz A, Jastrzebski T and Pawełczyk T: K-RAS point mutation, and amplification of C-MYC and C-ERBB2 in colon adenocarcinoma. Folia Histochem Cytobiol 42: 173-179, 2004.
27. Chen LT, Xu SD, Xu H, Zhang JF, Ning JF and Wang SF: MicroRNA-378 is associated with non-small cell lung cancer brain metastasis by promoting cell migration, invasion and tumor angiogenesis. Med Oncol 29: 1673-1680, 2012.
28. Deng H, Guo Y, Song H, Xiao B, Sun W, Liu Z, Yu X, Xia T, Cui L and Guo J: MicroRNA-195 and microRNA-378 mediate tumor growth suppression by epigenetical regulation in gastric cancer. Gene 518: 351-359, 2013.
29. Eichner LJ, Perry MC, Dufour CR, Bertos N, Park M, St-Pierre J and Giguère V: miR-378* mediates metabolic shift in breast cancer cells via the PGC-1β/ERRγ transcriptional pathway. Cell Metab 12: 352-361, 2010.
30. Lee DY, Deng Z, Wang CH and Yang BB: MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. Proc Natl Acad Sci USA 104: 20350-20355, 2007.
31. Li LH, Gao Q, Wang XY and Guo ZJ: miR-378 suppresses HBV-related hepatocellular carcinoma tumor growth by directly targeting the insulin-like growth factor 1 receptor. Zhonghua Gan Zang Bing Za Zhi 21: 609-613, 2013 (In Chinese).
32. Scapoli L, Palmieri A, Lo Muzio L, Pezzetti F, Rubini C, Girardi A, Farinella F, Mazzotta M and Carinci F: MicroRNA expression profiling of oral carcinoma identifies new markers of tumor progression. Int J Immunopathol Pharmacol 23: 1229-1234, 2010.
33. Mensink RP, Zock PL, Kester AD and Katan MB: Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. Am J Clin Nutr 77: 1146-1155, 2003.
34. Zhao L, Hu Y, Xu D and Cai K: Surface functionalization of titanium substrates with chitosan-lauric acid conjugate to enhance osteoblasts functions and inhibit bacteria adhesion. Colloids Surf B Biointerfaces 119: 115-125, 2014.
35. Fauser JK, Matthews GM, Cummins AG and Howarth GS: Induction of apoptosis by the medium-chain length fatty acid lauric acid in colon cancer cells due to induction of oxidative stress. Chemotherapy 59: 214-224, 2013.