MicroRNA profiling links miR-378 to enhanced adipocyte lipolysis in human cancer cachexia

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Submitted 3 May 2013; accepted in final form 5 December 2013

CANCER CACHEXIA (CC) is characterized by weight loss with reductions in skeletal muscle and adipose tissue mass, poor response to chemotherapy, and decreased survival (16). It affects around one-half of all patients with cancer and is more prominent as the disease progresses (6). Cachexia is also associated with nonmalignant conditions such as chronic infections (e.g., tuberculosis and human immunodeficiency virus), heart and renal failure, as well as chronic inflammatory diseases such as rheumatoid arthritis and Crohn’s disease (31). Results in recent years have demonstrated that adipose tissue from cancer patients with cachexia has a marked increase in catecholamine-stimulated lipolysis and fatty acid (FA) oxidation compared with weight-stable patients (2, 20, 31).

Fat cell lipolysis, the hydrolysis of triacylglycerol (TAG) to release FAs and glycerol, is a key function of adipocytes. In humans, lipolysis is primarily controlled by three hormones: catecholamines (stimulatory), natriuretic peptides (stimulatory), and insulin (inhibitory). Hydrolysis of adipocyte TAGs is regulated by two lipases: adipose triglyceride lipase (ATGL, encoded by PNPLA2) and the tri/diglyceride hormone-sensitive lipase (HSL, encoded by LIPE) (4). The lipolytic activity of ATGL requires the presence of the cofactor Comparative Gene Identification 58 (encoded by ABHD5). Besides lipases, lipid droplet-coating proteins are also important regulators of lipolysis (23). The most essential group is the perilipin family, including the phosphoprotein perilipin 1 (PLIN1, encoded by the gene with the same name), which is the most abundant lipid droplet-coating protein in adipocytes (29). Alterations in the expression of these lipolysis-regulating genes has previously been demonstrated in relation to cachexia (1, 12).

MicroRNAs (miRNAs) are small non-protein-coding RNAs that have well-established roles in regulating gene function (5). miRNAs regulate gene expression through binding to complementary sequences of target miRNAs, resulting in decreased mRNA levels (18). The miRNAs may act directly on the target genes or indirectly by first regulating transcription factors, which, in turn, control the expression of genes (40). Recent studies have shown that miRNAs play a role in lipid metabolism, glucose homeostasis, and the development of tissues involved in metabolism such as liver, muscle, and adipose tissue (22).

Global gene expression profiling of human adipose tissue previously identified the molecular pathways associated with weight loss in CC and found that the expression of genes regulating energy turnover, cytoskeleton, and extracellular matrix are altered in CC (11). However, the expression of miRNAs in CC has not been examined previously. In this work, we assessed whether CC is associated with changes in miRNA expression in abdominal subcutaneous adipose tissue and if the dysregulated miRNAs modulate fat cell lipolysis in vitro.

MATERIALS AND METHODS

Patients. Inclusion of patients has been described in more detail previously (11). Briefly, patients scheduled for gastrointestinal cancer operation between March 2004 and March 2008 were evaluated for the study, and patients who (i) were able to come to our clinical research laboratory for a fat needle biopsy and relevant clinical examinations, (ii) had not received prior anti-cancer (i.e., surgery, chemo- and/or radiotherapy) treatment, (iii) did not have clinical evidence of gastrointestinal obstruction, (iv) fit into the logistical scheme for scientific studies before operation or preoperative treatment, and (v) were willing participants were included (n = 53). None of the selected patients had jaundice. The patients were divided into two groups: (1) CC, which was defined as gastrointestinal cancer with self-reported

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unintentional weight loss of >5% of the habitual weight during the previous three months or >10% unintentional weight loss during the previous six months and 2) gastrointestinal cancer with no significant self-reported weight change during the last year (1). The miRNA array study included 10 patients with CC and 11 cancer patients with no significant self-reported weight change. The primary location of cancer in the cachexia group was pancreas (n = 8), stomach (n = 1), and liver metastases (n = 1) and in the weight-stable patients (n = 4) esophagus (n = 1), stomach (n = 1), liver metastases (n = 4), and gall bladder (n = 1). The study was approved by the Regional Ethics committee, and studies were performed in accordance with the statutes of the Declaration of Helsinki. The investigation was explained in detail to each patient, and written informed consent was obtained.

**Clinical evaluation.** Patients came to the laboratory for clinical examination following an overnight fast. Height, weight, body composition by bioimpedance using Quad Scan 4000 (Bodystat, Isle of Man, British Isles), and indirect calorimetry using Deltatrac (Datex-Engstroms, Helsinki, Finland) were determined. A venous blood sample was obtained for measuring lipids, glycerol, FA, albumin, and sensitive C-reactive protein (CRP), as previously described (1, 39). Nutritional status was assessed by a standardized oncology questionnaire termed Patient Generated Subjective Global Assessment (33).

**Cell culture.** Primary adipocyte culture for experimental studies was obtained from subcutaneous WAT from healthy men and women undergoing cosmetic liposuction. In this experimental group there was no selection for age, sex, or body mass index (BMI). Isolation of human adipocyte progenitor cells from subcutaneous WAT was performed exactly as described in detail (43). Briefly, WAT was washed, cut into small pieces, and digested with collagenase for 1 h at 37°C. The collagenase-treated cell suspension was centrifuged at 200 g for 10 min, and the supernatant, containing mature adipocytes and collagenase solution, was removed. The remaining stromal-vascular fraction (containing predipocytes and mesenchymal stem cells) was suspended in erythrocyte lysis buffer for 10 min, filtered through a nylon mesh, and centrifuged as above. The pellet was suspended in an inoculation medium DMEM-F-12 supplemented with 10% fetal bovine serum and 100 μg/ml penicillin-streptomycin and subsequently filtered through a 70-μm-pore size filter. The cells were plated at the density of 30,000–50,000 cells/cm² in inoculation medium to allow cell attachment. After 24 h the medium was changed to differentiation medium DMEM-F-12 supplemented with 15 mM HEPES, 100 μg/ml penicillin-streptomycin, 2.5 μg/ml amphotericin B, 66 mM human insulin, 1 mM triiodo-l-thyronine, 10 μg/ml human transferrin, 33 μM biotin, 17 μM panthenote, 100 mM cortisol, and 10 μM Rosiglitazone (BRL-49653)]. Rosiglitazone was included the first 3–6 days and then removed from the differentiation medium. The grade of fat cell differentiation was controlled under the microscope, and wells with a differentiation grade lower than ~80% were discarded.

Human adipose-derived stem cells (hADSCs) were isolated from subcutaneous WAT of a male donor (16 yr old, BMI 24 kg/m²). They were isolated, cultured, and differentiated to adipocytes using described protocols (15, 36, 46).

**RNA isolation, cDNA synthesis, and real-time quantitative PCR.** Total RNA was extracted from in vitro differentiated adipocytes and intact adipose tissue (300 mg) using the Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) or miRNeasy kit (Qiagen, Hilden, Germany), and 200–400 μg were treated with ribonuclease-free deoxyribonuclease. RNA concentration and purity were measured spectrophotometrically using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Lafayette, CO), and high-quality (total and small) RNA was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Total RNA was reverse transcribed using the Omniscript First-strand cDNA synthesis kit (Qiagen) and random hexamer primers (Invitrogen, Carlsbad, CA). RT-qPCR was performed using commercial Taqman probes or SYBR Green-based technology. PCR conditions and primers for detection of ILPE transcript have been described previously (13, 28). The 18S and low-density lipoprotein receptor-related protein 10 were used as reference genes with similar results. miRNA cDNA was synthesized using the TaqMan miRNA Reverse Transcription Kit and human Megaplex primer pool A without preamplification (Applied Biosystems, Foster City, CA). For miRNAs not included in the primer pool, specific probes designed by Applied Biosystems were used. We tested three transcripts as internal control for specific miRNA measurements: RNU48 (Applied Biosystems Assay 001006), miR-24 (000402), and miR-103 (000439) (32). Only expression of miR-103 was not associated with cachexia according to RT-qPCR data and was therefore used as internal control in subsequent quantification of expression of specific miRNA.

**Real-time PCR was performed on an iCycler IQ (Bio-Rad) using the amplification protocols provided by Applied Biosystems. The 2−ΔΔCt comparative method was used for data analysis (25).**

**Transfection of miRNA.** In vitro differentiated adipocytes were treated with 60 nM of miRIDIAN miRNA hairpin inhibitors or 40 nM intensities and present calls were generated in the miRNA QC tool by Affymetrix using default settings, i.e., BC-CG background adjustment, quantile normalization, and median polish summarization. Subsequent microarray analysis was limited to the 116 human miRNAs that were scored as present in ≥16 samples.

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**Transfection of miRNA.** In vitro differentiated adipocytes were treated with 60 nM of miRIDIAN miRNA hairpin inhibitors or 40 nM
of miRDIAN miRNA mimics (Thermo Fisher Scientific) and HiPerFect Transfection Reagent (Qiagen) at differentiation day 10-12 according to the manufacturers’ protocols. To rule out unspecified effects, control cells were transfected with hairpin inhibitor or mimics nontargeting negative controls (Thermo Fisher Scientific). The cells were incubated for 48–72 h at which time in vitro lipolysis was assessed and RNA or proteins were collected. Transfection efficiency was evaluated by qRT-qPCR using miRNA probes (Applied Biosystems) and miScript Primer Assays (MS00006909; Qiagen) with the SNORD68 (small nucleolar RNA, C/D box 68, MS0003712, Qiagen) or RNU48 genes as internal controls.

**Lipolysis ex vivo in isolated adipocytes.** Fat cells were isolated from adipose tissue pieces from clinical samples using collagenase digestion as previously described (35). Mean fat cell weight and volume were determined and lipolysis experiments conducted as described (26). Briefly, diluted cell suspensions (2% vol/vol) were incubated in duplicate for 2 h with air as the gas phase at 37°C in Krebs-Ringer phosphate buffer (pH 7.4) supplemented with glucose (8.6 mmol/l), ascorbic acid (0.1 mg/ml), and BSA (20 mg/ml) without (basal) or with increasing concentrations of norepinephrine (10⁻¹² to 10⁻⁴ mol/l). After the incubation period, glycerol release was determined as an index of lipolysis. Results were expressed as amount of glycerol release per 2 h per 10⁶ fat cells. Half-maximum effective norepinephrine concentration (EC₅₀) was determined and turned into a pD₂ value (-log₁₀ EC₅₀). Maximum effect was calculated as lipolysis at maximum effective concentration.

**Lipolysis in in vitro differentiated adipocytes.** For assessment of lipolysis in the in vitro differentiated adipocytes, cells were incubated for 3 h in DMEM-F-12 medium supplemented with 20 g/l of BSA and increasing concentrations of norepinephrine. Glycerol in media was measured using Free Glycerol Reagent (Sigma Aldrich, St. Louis, MO) and Amplex UltraRed (Invitrogen) according to the manufacturer’s instructions. Amplex Ultra Red was diluted 100-fold in Free Glycerol Reagent, mixed with 20 μl of conditioned medium in a 96-well plate, and incubated at room temperature for 15 min, and fluorescence was measured (excitation/emission 530/590) using an Infinite M200 plate reader (Tecan Group, Männedorf, Switzerland).

**Analysis of protein expression.** Cells were lysed in RIPA buffer as described previously (16). Total protein (20–30 μg) was separated by SDS-PAGE, and Western blot was performed according to standard procedures. The membranes were blocked in 3% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, UK), primary antibodies against HSL and ATGL were from Cell Signaling Technology (Danvers, MA), and antibodies against PLIN1 were from Progene (Heidelberg, Germany). β-Actin (Sigma-Aldrich) was used as a loading control. Secondary antibodies mouse/rabbit IgG-horse-radish peroxidase were from Sigma-Aldrich. Antibody-antigen complexes were detected by chemiluminescence using an ECL Select Western Blotting Detection Kit (GE Healthcare).

**Staining procedures.** Adipocytes were transfected with miR-378 inhibitor for 48 h and then fixed with 5% paraformaldehyde. Subsequently, nuclei were stained with Hoechst 33342 (2 μg/ml) and intracellular lipids with BODIPY 493/503 (0.2 μg/ml) in PBS in the dark at room temperature for 30 min, and thereafter washed two times with PBS. Accumulation of intracellular lipid droplets and cell number (as indicated by stained nuclei) were measured using the Acumen eX3 imager (TTP Labtech, Hertfordshire, UK), and total fluorescence for neutral lipids was normalized to the amount of nuclei in each well.

**miRNA target prediction analysis.** We used the miWalk web tool to predict the targets of the selected miRNAs (14). A list of lipolysis-related genes (41) was imported into miWalk, and predictions of miRNAs were performed using the default settings. Four additional algorithms were performed for filtering purposes, each one using different sets of properties among mRNA-miRNA targeting (TargetScan, miRanda, miRDB, and RNA22). Predicted miRNA targets were considered significant when predicted by all five algorithms.

**Statistical analysis.** Data are shown as means ± SD. Results were analyzed by paired or unpaired t-test, chi square (for gender), or Mann-Whitney, and correlations were determined by simple and multiple regression. Microarray results were analyzed with significance analysis of microarrays (42). SAM adjusts for the multiple comparisons caused by the presence of probe sets for several miRNAs on the microarrays. The software assigns a score to each miRNA on the basis of change in expression relative to the SD of repeated measurements. For miRNAs with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the false discovery rate (FDR). One thousand permutations were used in the numerical analysis. Two-group unpaired comparison was applied. Signal values from the 116 human miRNAs that were scored as present in adipose tissue were included in the analysis.

**RESULTS**

**Clinical observations.** Relevant clinical data are summarized in Table 1. Body fat mass was markedly decreased in subjects with CC compared with weight-stable cancer patients, whereas lean body mass, age, and gender distribution did not differ between the two groups. The difference in fat, but not lean body mass between groups, suggests that the CC patients had lost adipose tissue but not muscle mass, most probably due to the fact that they were recently diagnosed with their malignant disease. Subjects in the CC group had a tumor stage of 3 (range 1–4) vs. 4 (range 0–4) in the weight-stable group (P = 0.04) and self-reported a mean decrease in their habitual weight of about 6.5% in the last three months before diagnosis. As expected, CC was accompanied by signs of systemic inflammation, i.e., elevated CRP. Lipid mobilization and oxidation were increased in cachexia as evidenced by differences in plasma concentrations of glycerol and FAs, and in respiratory

<table>
<thead>
<tr>
<th>Measure</th>
<th>Cancer Cachexia</th>
<th>Cancer Weight Stable</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, M/F</td>
<td>7/3</td>
<td>6/5</td>
<td>0.72</td>
</tr>
<tr>
<td>Age, yr</td>
<td>64 ± 6</td>
<td>63 ± 7</td>
<td>0.005</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.0 ± 2.6</td>
<td>24.6 ± 3.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>18.3 ± 5.6</td>
<td>28.1 ± 7.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Body fat, kg</td>
<td>12.0 ± 3.7</td>
<td>20.5 ± 5.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>55.5 ± 14.6</td>
<td>53.8 ± 14.1</td>
<td>0.80</td>
</tr>
<tr>
<td>Weight loss, % Habitual wt</td>
<td>6.5 ± 3.5</td>
<td>0.7 ± 3.2</td>
<td>0.001</td>
</tr>
<tr>
<td>P-glucose, mmol/l</td>
<td>6.0 ± 1.0</td>
<td>6.4 ± 1.9</td>
<td>0.52</td>
</tr>
<tr>
<td>P-triglycerides, mmol/l</td>
<td>0.96 ± 0.3</td>
<td>1.4 ± 0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>P-cholesterol, mmol/l</td>
<td>4.4 ± 1.1</td>
<td>5.2 ± 1.1</td>
<td>0.12</td>
</tr>
<tr>
<td>S-albumin, g/l</td>
<td>34.2 ± 4.2</td>
<td>39.0 ± 2.4</td>
<td>0.004</td>
</tr>
<tr>
<td>S-CRP, mg/l</td>
<td>15 ± 20</td>
<td>2 ± 1</td>
<td>0.04</td>
</tr>
<tr>
<td>P-glycerol, μmol · l⁻¹, kg body fat⁻¹</td>
<td>9.9 ± 5.6</td>
<td>4.2 ± 2.2</td>
<td>0.005</td>
</tr>
<tr>
<td>P-fatty acids, mmol · l⁻¹, kg body fat⁻¹</td>
<td>0.08 ± 0.03</td>
<td>0.04 ± 0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>PG-SGA score, points</td>
<td>8 (4–17)</td>
<td>2 (1–3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor stage, points</td>
<td>3 (1–4)</td>
<td>4 (0–4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Respiratory quotient, V̇O₂/V̇CO₂</td>
<td>0.81 ± 0.02</td>
<td>0.84 ± 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Resting energy expenditure, kcal/day</td>
<td>1.596 ± 0.239</td>
<td>1.653 ± 0.324</td>
<td>0.69</td>
</tr>
<tr>
<td>Fat cell volume, pl</td>
<td>308 ± 128</td>
<td>510 ± 159</td>
<td>0.006</td>
</tr>
<tr>
<td>Fat cell no. x 10⁶</td>
<td>4.8 ± 1.7</td>
<td>4.6 ± 0.9</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Values are means ± SD or mean (range). M; male; F; female; P; fasting plasma; S; fasting serum; CRP; PG-SGA, Patient Generated Subjective Global Assessment. CRP was log₁₀ transformed before comparison. Groups were compared by unpaired t-test, chi square (for gender), or Mann-Whitney (for PG-SGA and tumor stage).
miRNA expression in cachexia. Global miRNA levels in abdominal subcutaneous adipose tissue of CC and weight-stable cancer patients were determined. Fifteen of the 116 adipose-expressed miRNAs displayed a significant difference in expression between the two groups according to t-test with nominal P value (<0.05, Table 2). Nine of these miRNAs displayed a significant difference between CC and weight-stable cancer patients with FDR 6.8% according to SAM, which takes into account multiple comparisons. We were unable to obtain a 5% FDR in the graphical interface of SAM. Using RT-qPCR, we aimed to confirm the 15 miRNAs with cachexia-associated expression according to t-test. This was motivated by the limited power of our study due to the small sample size and the fact that t-test and SAM generated overlapping results. However, four miRNAs could not be amplified by RT-qPCR either due to failure to design an assay (n = 3) or because the miRNA had been withdrawn from public databases (n = 1). One additional miRNA, miR-191, was not followed up by RT-qPCR due to small fold change, 0.93, between groups. It is our experience that a fold change of at least 20% on microarray is necessary for successful confirmation. We could validate that the expression of 5 of the 10 remaining miRNAs displayed a significant and directionally consistent association with cachexia according to RT-qPCR. Expression of miR-483–5p/-23a/-744/-99b/-378 was downregulated in CC, whereas miR-378 was significantly upregulated (Table 2).

Cachexia-dysregulated miRNAs and lipolysis. To explore the association of miR-483–5p/-23a/-744/-99b/-378 with catecholamine-stimulated lipolysis, we correlated adipose tissue miRNA expression according to RT-qPCR with ex vivo fat cell lipolysis in our patient samples. Whereas the expression of the four downregulated miRNAs did not correlate with lipolysis ex vivo (data not shown), there was a strong positive correlation between miR-378 expression and catecholamine-stimulated lipolysis (r = 0.839, P < 0.0001, Fig. 1). The correlation between miR-378 and lipolysis remained significant after adjusting for BMI (P = 0.0055). Further functional experiments were therefore focused on this miRNA.

To examine whether miR-378 could regulate adipocyte lipolysis in vitro, primary human adipocytes obtained from multiple donors were transfected with a miR-378 hairpin inhibitor, and 48 h posttransfection basal and norepinephrine-stimulated lipolysis were assessed. Expression of miR-378 was efficiently attenuated by the miR-378 hairpin inhibitor, with almost 90% downregulation compared with the negative control (Table 3). Basal (non-hormone-stimulated) lipolysis rates were not affected by reduced expression of miR-378 (Fig. 2A). However, efficient attenuation of miR-378 expression led to a significant downregulation of norepinephrine-induced lipolysis (P = 0.02, Fig. 2A). Similar effects were observed in multiple experiments using in vitro differentiated adipocytes derived from hADSCs cells of one donor (Fig. 2B). Conversely, overexpression of miR-378 in these cells resulted in increased stimulated lipolysis (Fig. 2B).

Specificity of mir-378 inhibition in in vitro differentiated adipocytes. To demonstrate the specificity of miR-378 inhibition, we quantified expression of two other cachexia-associated miRNAs (miR-744 and -99b) in differentiated adipocytes.
Table 3. Specificity of miR-378 inhibition

<table>
<thead>
<tr>
<th>miRNA or Gene ID</th>
<th>Fold Change</th>
<th>SD</th>
<th>t-Test P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected miRNAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-378</td>
<td>0.12</td>
<td>0.021</td>
<td>1.998\textsuperscript{-5}</td>
</tr>
<tr>
<td>miR-744</td>
<td>0.94</td>
<td>0.08</td>
<td>0.101</td>
</tr>
<tr>
<td>miR-99b</td>
<td>0.96</td>
<td>0.06</td>
<td>0.281</td>
</tr>
<tr>
<td>Unrelated miRNAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTN1</td>
<td>1.05</td>
<td>0.11</td>
<td>0.551</td>
</tr>
<tr>
<td>CCL2</td>
<td>1.11</td>
<td>0.26</td>
<td>0.380</td>
</tr>
<tr>
<td>Unrelated genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDCD4</td>
<td>0.94</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>Apoptosis-related genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDCD4</td>
<td>0.92</td>
<td>0.13</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Data are expressed as a relative fold change miRNA vs. negative (nontargeting) control. Effects of miR-378 inhibition on expression of cachexia-associated miRNAs and genes were investigated by transfecting human in vitro differentiated adipocytes with miR-378 inhibitor and assessing miRNA/mRNA expression (\(n=6-7\) experiments). ACTN1, actinin, \(\alpha_1\); CCL2, chemokine (C-C motif) ligand 2; PTEN, phosphatase and tensin homolog; PDCD4, programmed cell death 4 (neoplastic transformation inhibitor).

transfected with miR-378 inhibitor. Although miR-378 was efficiently downregulated, expression levels of miR-744 and -99b were not affected (Table 3). Additionally, we determined the expression of two other adipose genes. Specifically, ACTN1 (encoding the protein actinin \(\alpha_1\)) has previously been identified by us as a cachexia-regulated gene (11), whereas chemokine (C-C motif) ligand 2 (CCL2) is an important inflammatory mediator released by adipocytes. The levels of ACTN1 and CCL2 were not affected by in vitro inhibition of miR-378. To assess if miR-378 inhibition could affect apoptosis-related genes, mRNA expression of two well-described apoptotic genes was quantified. Expression of PTEN (phosphatase and tensin homolog) (30) and PDCD4 (programmed cell death 4, neoplastic transformation inhibitor) (45) was not affected by miR-378 downregulation (Table 3). Moreover, the content of neutral lipids was not affected by altered miR-378 expression (ratio miR-378 inhibitor vs. negative control = 0.92 ± 0.32; \(P = 0.43\)). In summary, these data indicate that the miR-378 inhibitor reagent only affected the expression of its target miRNA and did not influence the levels of a set of unrelated miRNAs and genes indicating specificity of the miR-378 inhibitor reagent.

**miR-378 regulates expression of key lipolysis genes.** To further explore the effects of miR-378 on adipocyte lipolysis, we performed mRNA expression analysis of genes encoding key proteins involved in lipolysis in cells transfected with the miR-378 hairpin inhibitor. The mRNA levels of LIPE, PNPLA2, and PLIN1 were significantly downregulated in miR-378 inhibitor-treated samples (Table 4). In contrast, ABHD5 expression was not significantly affected. The levels of HSL (LIPE), PLIN1, and ATGL (PNPLA2) proteins mirrored the mRNA expression results (Table 4 and Fig. 3). Approximately 90% downregulation of miR-378 (Table 3) was associated with 16–35% downregulation of target genes (Table 4). Next, we explored if lipolysis-related genes were direct targets of cachexia-associated miRNAs. Bioinformatic analysis failed to identify that LIPE, PLIN1, and PNPLA2 or other lipolysis-related genes (41) are predicted targets for direct action by miR-378 [Supplemental Table 1 (Supplemental data for this article may be found on the *American Journal of Physiology: Endocrinology and Metabolism* website)]. Finally, the other cachexia-associated miR-483-5p/-23a/-744/-99b did not target any of the established lipolysis genes (Supplemental Table 1).

**DISCUSSION**

miRNAs have been extensively studied in cancer, but to our knowledge their impact in CC has not been determined previously. Here we report five miRNAs displaying altered expression in subcutaneous adipose tissue in cancer subjects with cachexia compared with weight-stable cancer patients. One of these, miR-378, displays an expression that correlates positively with hormone-stimulated lipolysis in adipocytes. Furthermore, attenuation of miR-378 expression in human adipocytes differentiated in vitro results in reduced catecholamine-stimulated lipolysis possibly secondary to reduced gene expression of key regulatory elements, including HSL (LIPE), PLIN1, and ATGL (PNPLA2).

In different experimental models, miR-378 has previously been shown to increase during adipogenesis and to stimulate the expression of lipogenic genes (17), suggesting a role in fat cell physiology. In rodents, inhibition of miR-378 stimulates whole body energy expenditure and FA oxidation, which has been linked to altered hepatic gene expression (9). These results suggest that miR-378 has complementary regulatory functions in lipid metabolism in different organs. These might...
Effects of miR-378 on mRNA and protein levels of lipolysis-related genes

<table>
<thead>
<tr>
<th>Protein or Gene ID</th>
<th>mRNA</th>
<th>SD</th>
<th>t-Test P Value</th>
<th>Protein</th>
<th>SD</th>
<th>t-Test P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSL (LIPE)</td>
<td>0.78</td>
<td>0.15</td>
<td>0.007</td>
<td>0.77</td>
<td>0.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>ATGL (PNPLA2)</td>
<td>0.65</td>
<td>0.17</td>
<td>0.001</td>
<td>0.84</td>
<td>0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>CGI-58 (ABHD5)</td>
<td>0.71</td>
<td>0.30</td>
<td>0.0022</td>
<td>0.087</td>
<td>0.14</td>
<td>0.044</td>
</tr>
<tr>
<td>Perilipin (PLIN1)</td>
<td>0.84</td>
<td>0.16</td>
<td>0.028</td>
<td>0.88</td>
<td>0.14</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Data are expressed as a relative fold change miRNA vs. negative (nontargeting) control. Effects on lipolysis-related genes by miR-378 were investigated by transfecting human in vitro differentiated adipocytes with miR-378 inhibitor and assessing miRNA and protein expression of the indicated genes (n = 6–7 experiments).

Table 4. Effects of miR-378 on mRNA and protein levels of lipolysis-related genes

represent a general weight-regulating pathway in response to altered energy balance or weight change, since miR-378 has been reported to be downregulated in adipose tissue from obese (3, 10).

Our results for LIPE and PNPLA2 are of particular interest. Increased catecholamine-stimulated lipolysis in CC has previously been attributed to increased HSL (LIPE) levels (1), and ATGL (PNPLA2)-deficient mice resisted adipose tissue lipolysis and CC (12). However, neither LIPE and PNPLA2 nor 60 other known lipolysis genes were confidently predicted as direct targets of miR-378. This indicates that there are likely additional steps between miR-378 and PLIN1, LIPE and PNPLA2 that in turn may explain why strong, almost 90%, downregulation of miR-378 expression had a limited impact on lipolysis and expression of lipolysis-regulating genes. However, mapping of these pathways necessitates the screening of all genes present in adipocytes and was beyond the scope of the present work. It is important to stress that it is difficult to directly translate in vitro-induced changes in miRNA expression to an in vivo situation. Nevertheless, the fact that miR-378 levels were ~30% higher in adipose tissue of cachectic subjects suggests that even smaller alterations in miRNA expression over longer periods of time could have an impact on adipose lipolysis.

In this study we only observed effects of miR-378 expression on hormone-stimulated lipolysis. It is well established that effects on basal lipolysis are observed when LIPE, PNPLA2, and/or PLIN1 levels are altered. However, this is primarily seen when the levels of either corresponding proteins are substantially reduced (i.e., >50% at the protein level), see, for instance, Ref. 38. The rather modest effects on protein expression of these lipolysis regulators observed in vitro in this study could possibly explain the lack of effect on basal lipolysis.

Interestingly, miR-378 has been suggested as a marker for specific cancer forms, e.g., gastric cancer (24). Could miR-378 constitute a functional link between cancer and cachexia? This could be investigated by examining miR-378 levels in relation to the severity of CC and disease outcome in larger patient materials. Unfortunately, we do not have access to such cohorts.

Only 5 out of 10 miRNAs regulated by cachexia according to the microarray could be confirmed by RT-qPCR. There are several explanations for this, e.g., the small sample size, high FDR in primary analysis, and small fold changes. Although miRNA differences between cachectic and weight-stable control cancer patients were small, they were in the same range as we previously observed for obesity-associated miRNAs comparing obese vs. nonobese (3). We therefore believe these changes are clinically relevant. Four of the five miRNAs were not associated with lipolysis in the clinical cohort, i.e., miR-483–5p/23a/-744/-99b. However, this does not exclude that they may be involved in other pathways of relevance for CC. For example, miR-483–5p has previously been shown to control the expression of SOCS3 and IGF2, genes implicated in regulation of leptin signaling and adipogenesis, respectively (8, 27). PGC1α, which encodes the transcriptional cofactor peroxisome proliferator-activated receptor-γ coactivator-1α, is an established inducer of mitochondrial β-oxidation and electron transport and is a direct target of miR-23a (44). Furthermore, miR-23a regulates glucose transport (34) and interferes with mitochondrial function (37). Interestingly, we have previously shown that CC is associated with increased expression of genes (cytochrome c oxidase subunit 8A, cytochrome b5 type A, cytochrome c-1, pyruvate carboxylase, branched-chain amino-transferase) related to energy expenditure in human adipose tissue (11).

Finally, the present work focused on effects of miR-378 in human adipocytes. Unfortunately, the limited amount of tissue available from our cancer cohort precluded studies of different cell fractions within adipose tissue. Therefore, at present, we cannot exclude that miR-378 (and other described miRNAs) may display cell-specific differences in expression and/or play different functional roles in adipose tissue of cancer subjects with or without cachexia.

Fig. 3. MiR-378 regulates lipolysis-related proteins. In vitro differentiated human adipocytes were transfected with inhibitor of miR-378 for 48–72 h, and cells were collected for protein analysis (n = 6). Representative Western blots for hormone-sensitive lipase (HSL), perilipin 1 (PLIN1), and adipose triglyceride lipase (ATGL) are shown. Quantification of protein data obtained from multiple experiments is shown in Table 4. Reassembly of noncontiguous gel lanes between negative control and miR-378-treated samples is indicated by a line.
In conclusion, we have identified miRNA-378 as a novel regulator of catecholamine-stimulated lipolysis in fat cells with potential relevance as a drug target for human CC.

ACKNOWLEDGMENTS

We thank Eva Sjölin, Elisabeth Dunger, Gaby Aaström, Kerstin Wählén, Britt-Marie Leijonhufvud, Katarina Hertel, and Yvonne Widing for technical assistance. We also thank Jöming Liu at the Department of Biosciences and Nutrition, Karolinska Institutet for help with the Acumen reader.

GRANTS

This work was supported by several grants from the Swedish Research Council (P. Arner, I. Dahlman, M. Ryden), the Swedish Diabetes Foundation (I. Dahlman, P. Arner), the Diabetes Program at Karolinska Institutet (P. Arner), the Swedish Society of Medicine (A. Kulyté), Novo Nordisk Foundation (M. Ryden), Tore Nilsson foundation (A. Kulyté), Stiftelsen för Gamla Tjänarinnor (A. Kulyté), Åke Wiberg foundation (A. Kulyté), and The Swedish Cancer Foundation (M. Ryden).

DISCLOSURES

Informed consent was obtained from the subjects. Authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS


REFERENCES


