

Figure 3. A multi step process of metastasis and therapeutic targets of NF- κ B inhibitors A multi step process of metastasis (including invasion, transport, arrest, adherence, extravasation, and tumor cell proliferation) proceeded between weeks 16 and 22. Therefore, using this model, chemical substances could be applied in the intervening period to investigate modifying factors, particularly those leading to inhibition of lung metastasis formation. NF- κ B inhibitors have the potential to inhibit lung metastasis of rat HCCs *in vivo*, with PTX being the most promising candidate. They may interfere with attachment of tumor cells to the vascular endothelium at metastatic sites, and decrease of VEGF-A188 may play an important role.

of malignant tumors which metastasize to lung, and this model should be applicable not only for the elucidation of mechanisms underlying metastasis, but also to test anti-metastatic agents.

ASP, but not IM, has the potential to inhibit lung metastasis by rat HCC *in vivo*, the mechanism apparently involving neither inhibition of cell proliferation nor detachment from primary tumors. Inhibition of attachment to the vascular endothelium in the lung is more likely to be the mechanism responsible for the suppression of lung metastasis formation by ASP. NF- κ B inhibitors have the potential to inhibit lung metastasis of rat HCCs *in vivo*, with PTX being the most promising candidate. They may interfere with attachment of tumor cells to the vascular endothelium at metastatic sites, and decrease of VEGF-A188 may play an important role (Figure 3).

This *in vivo* model for induction of rat highly metastatic hepatocellular carcinomas is clearly a useful tool for the assessment of the efficacy of therapeutic treatments for metastasis formation and for analysis of individual steps in the metastatic process.

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Promotive effects of cell proliferation and chromosomal instability induced by tribbles-related protein 3 in mouse mammary tumor cells

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Abstract. Tribbles-related protein 3 (TRB3) has been shown to be a crucial modulator of tumorigenesis. However, the precise role and the functional morphology of TRB3 are not clearly understood. To elucidate these enigmas we established the cell line, M2TRB3, by introducing the human TRB3 gene and protein in Cl66M2 (M2) mouse mammary tumor cells. This cell line stably expressed the TRB3 gene and protein. After 72 h of cell culture, there was a 34% increase in the growth of M2TRB3 cells compared to the control M2 mock cells. The mean volume of the tumors originating from the M2TRB3 cells was significantly increased by 38% when compared to the mean volume of the M2 mock tumors, and the proliferating cell nuclear antigen (PCNA) labeling index in the M2TRB3 tumors was higher when compared to that of the M2 and M2 mock cells. In the tumor tissue samples, the mean diameter of nuclei in the M2TRB3 tumor cells ($9.4 \pm 0.3 \mu\text{m}$) showed a significant increase compared to that of the M2 mock tumor cells ($7.0 \pm 0.2 \mu\text{m}$). M2TRB3 cells also showed a marked increase in the population of tetraploid or octaploid nuclei compared to M2 mock cells bearing mainly either diploid or tetraploid nuclei. Western blot analysis revealed the overexpression of cyclin B1 and cyclin D1 in M2TRB3 cells when compared to that in the M2 mock cells. These novel findings provide further evidence that TRB3 promotes cell proliferation and chromosomal instability by causing polyploidization during development.

Introduction

Tribbles-related protein 3 (TRB3, also known as NIPK, SKIP3) is a mammalian homologue of the *Drosophila Tribbles* gene, and this gene has been identified as an inhibitor of mitosis that regulates cell proliferation, migration and morphogenesis during development (1-3). Among tribbles homologues TRB1, TRB2 and TRB3, TRB3 is the most recently defined family of pseudokinases that contain a serine/threonine kinase catalytic domain but lack an ATP binding site or one of the conserved catalytic motifs essential for kinase activity (4). The interacting partners of TRB3 range from transcription factors, ubiquitin ligase, bone morphogenetic protein (BMP) type II receptor to members of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways. By interacting with these proteins, it coordinates crucial cellular processes, including glucose/lipid metabolism, apoptosis, adipocyte differentiation, cell stress and regulation of collagen expression (5-9). We previously demonstrated that TRB3 is induced by C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) to regulate their function and endoplasmic reticulum (ER) stress-induced cell death (10) and that TRB3 also regulates the stability of cell division cycle 25A (Cdc25A), an essential activator of cyclin-dependent kinases (CDKs) (11).

Recent studies indicate that the three mammalian tribbles homologues are crucial modulators of tumorigenesis. For instance, both TRB1 and TRB2 are involved in myeloid leukemogenesis (12,13). TRB3 is highly expressed in a wide range of human carcinoma cell lines and in several types of human carcinomas (4,14). However, a precise role of TRB3 in tumorigenesis remains unknown. The aim of the present study was to examine whether the introduction of the human *TRB3* gene into mouse mammary tumor cells affects *in vitro/in vivo* growth and chromosomal stability during cell division of tumor cells.

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Materials and methods

Cell culture. The human embryonic kidney cell line 293 purchased from the American Type Culture Collection (ATCC,

Manassas, VA, USA) and the human hepatocellular carcinoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD, USA) in a humidified incubator with 5% CO₂ at 37°C. The murine mammary tumor cell line Cl66M2 (M2) was generously provided by Dr Rakesh K. Singh (University of Nebraska Medical Center, Omaha, NE, USA) (18) and cultured in DMEM supplemented with 5% FBS in a humidified incubator with 5% CO₂ at 37°C.

Construction of the expression vector. The TRB3 flag-tagged expression vector was constructed by ligating the full length human TRB3 cDNA into *Bam*HI and *Xho*I restriction sites of pcDNA3.1-Hygro (Life Technologies, Inc.) (9). The construct was verified by sequencing.

Preparation of a cell line that stably expresses the TRB3 gene. The expression vector pcDNA3.1-Hygro-flag-human TRB3 was transfected into Cl66M2 cells using Lipofectamine 2000 reagent (Life Technologies, Inc.). This cell line was termed M2TRB3. After transfection, the clone of the cells stably expressing M2TRB3 was selected by a limiting-dilution method in culture media supplemented with hygromycin. The cells transfected with the empty vector were also prepared as the control (M2mock). In M2TRB3 and M2mock cells, the levels of mRNA and protein expression were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot assays.

In vitro cell proliferation assay. These assays were performed as described previously by us (15). Two murine mammary tumor cell lines M2TRB3 and M2mock were plated into 6-well 35-mm diameter culture plates (1.0x10⁴ cells/well) in DMEM containing 10% FBS. Cells were starved in DMEM containing 0.5% FBS for 48 h. After starvation, the culture media were removed and cells were grown in DMEM containing 10% FBS for the indicated time course (0-72 h). The cells were washed twice with phosphate-buffered saline (PBS), harvested, resuspended in 1 ml PBS and the number of cells was determined using a hemocytometer Burkert-Turk (Erma Inc., Tokyo, Japan). Each assay was repeated more than three times to confirm the results. The number of cells was plotted on a time-response curve as indicated in the figures.

Tumor xenograft assay. Male four-week-old BALB/cSlc-nu/nu mice obtained from Japan SLC, Inc. (Shizuoka Japan) were used. M2, M2TRB3 and M2mock cells (1.0x10⁶/200 μ l) were subcutaneously inoculated into the right lower flank of the mice. Tumor diameters (mm) and body weight (g) were recorded twice weekly. The tumor volume (mm³) was calculated by the formula: Volume = L x D x H x π /6, where L is the length, D is the depth, and H is the height. At 35 days after inoculation, all mice were euthanized and complete autopsies were performed. Animal experiments were conducted in accordance to the regulations specified by the Institutional Animal Use and Care Committee of Nagoya City University.

Immunohistochemistry and measurement of proliferating cell nuclear antigen (PCNA) labeling index. These assays were

performed using an established method as described previously by us (16). Paraffin sections (3- μ m) were prepared to include tumors resected from the lower flank of each mouse. These sections were treated in 3% H₂O₂ for 10 min to block the endogenous peroxidase activity. For antigen retrieval, the sections were brought to boiling in 0.1 M citrate buffer, pH 6.0. Sections were incubated with a primary antibody of PCNA (1:500 dilution) (sc-56; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 60 min. After incubation with the secondary antibody, sections were then stained using an ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. The PCNA labeling index was determined by calculating the ratio of PCNA-positive nuclei/total number of nuclei counted. Ten high power fields (x400) per tumor were examined, and >300 cells were counted in each tumor. In M2TRB3 and M2mock tumors, the longest diameter of the nucleus was determined by image analysis using Olympus DP70 system (Olympus Corp., Tokyo, Japan). Four high power fields (x400) per tumor were examined and more than 100 nuclei were counted in each tumor.

Flow cytometric analysis. These assays were performed as previously described (16). M2TRB3 and M2mock cells (7.5x10⁴ cells/plate) were plated onto 9-cm culture dishes in DMEM plus 10% FBS and grown to yield 50-60% confluence. To synchronize cells at the G0/G1 phase, they were starved by culturing in DMEM plus 0.5% FBS for 48 h. After starvation, cells were then grown in DMEM plus 10% FBS for 72 h. Adherent cells were washed twice with PBS, fixed with 5 ml 70% ethanol, centrifuged, resuspended in 400 μ l PBS containing 2 mg/ml RNase (Nacalai Tesque, Inc., Kyoto, Japan), and stained with 400 μ l of 0.1 mg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) in the dark for 30 min or overnight. The cell suspension was filtered through a 60- μ m nylon filter (Ikemoto Scientific Technology Co., Ltd., Tokyo). Samples of 10,000-20,000 cells were then analyzed for cell cycle phase distribution and ploidy status using a FACSCalibur™ instrument, and the data were analyzed with the CellQuest computer program (both from Becton-Dickinson, Franklin Lakes, NJ, USA) as described in a previous study (16). Cells were harvested just after starvation (0 h) and 72 h after starvation as described in Table I and Fig. 5. Each assay was repeated more than three times to confirm the results.

RT-PCR assays. These assays were conducted using previously established procedures (16). Total RNA was extracted from each cell line grown in 9-cm culture dishes using Isogen (Nippon Gene, Toyama, Japan). The reaction mixture contained 4 μ g of total RNA, 1 μ l of 10 mM dNTP, 1 μ l of random primers (both from Life Technologies, Inc.) and 7 μ l of distilled water. The reaction mixture was incubated at 65°C (5 min) for denaturation, chilled on ice for 1 min and 4 μ l of 5X RT buffer (Life Technologies, Inc.), 1 μ l of 0.1 M dithiothreitol (DTT), 1 μ l of the RNaseOut and 1 μ l of Superscript® III Reverse Transcriptase (both from Life Technologies, Inc.) were added. After the addition of these reagents, the reaction mixture was incubated at 50°C (60 min) for random primer annealing and 70°C (15 min) for cDNA preparation. One microliter of the reaction mixture was then used for PCR. The primer sequences used in this study were as follows: human TRB3-specific

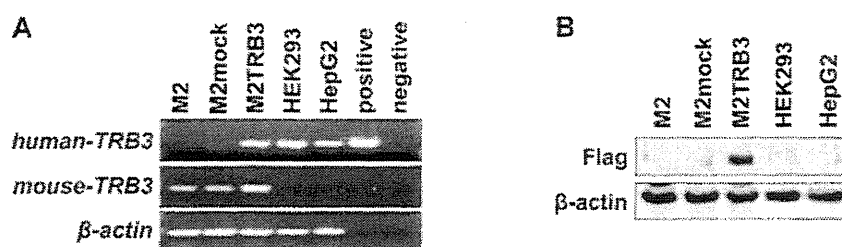


Figure 1. Expression status of exogenous human TRB3 and endogenous mouse TRB3 in the indicated cell lines. (A) Representative results of the RT-PCR analysis. The band in the positive control was derived from DNA fragments that were PCR-amplified using pcDNA3.1-Hygro-flag-human TRB3 vector as a template and the pairs of gene-specific primers listed in Materials and methods. No band was noted in the negative control since the empty vector (pcDNA3.1-Hygro) that does not carry specific sites for the primers was used as a template for PCR. Bands of β -actin were not observed in the positive and negative control samples due to the same reason as the negative control. (B) Representative results of the western blot analysis. The exogenous human TRB3 (Flag) protein was detected in the M2TRB3 cells.

primer set, hTRB3F (5'-CAAGTCGCTCTGAAGGTTCC-3') and hTRB3R (5'-CCATCCTACTCTGGCAAAGC-3'), mouse TRB3-specific primer set, mTRB3F (5'-CAAGTCGCTCTGAAGGTTCC-3') and mTRB3R (5'-CCATCCTACTCTGGCAAAGC-3'), respectively.

β -actin-specific DNA fragments from the same RNA samples were amplified and served as internal controls. Primers actinF (5'-CCGTAAAGACCCTCTATGCCAACA-3') and actinR (5'-CGGACTCATCGTACTCCTGCTT-3') were used for amplification of β -actin. PCR was conducted for 26-30 cycles in an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 60°C for primer annealing, and 1 min at 72°C for extension. After PCR amplification, the DNA fragments were stained with ethidium bromide and analyzed by 2% agarose gel electrophoresis. The results were confirmed by repeating the experiments.

Western blot assays. These assays were conducted according to previously established procedures (17). The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, and 1% Triton X-100]. The lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12.5%), transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore Corp., Bedford, MA, USA) and probed with the antibodies. The primary antibodies used in the present study were anti- β -actin monoclonal antibody (AC-15) (Sigma-Aldrich), anti-cyclin B1 monoclonal antibody (sc-245) (Santa Cruz Biotechnology Inc.), anti-Cdc2 monoclonal antibody (sc-54), anti-Cdk2 polyclonal antibody (sc-163), anti-Cdk4 polyclonal antibody (sc-260), anti-TRB3 polyclonal antibody (sc-34211), anti-cyclin D1 monoclonal antibody (556470; Becton-Dickinson), and anti-Flag monoclonal antibody (018-22381) (Wako Pure Chemical Industries, Ltd.). The immunoreactive proteins were visualized using ImmunoStar Zeta (Wako Pure Chemical Industries, Ltd.) and light emission was quantified with Light Capture (ATTO Corp., Tokyo, Japan). Each assay was repeated more than three times to confirm the results.

Statistical analysis. Differences in the number of cells, tumor volume, PCNA labeling index, and rate of DNA ploidy between cell lines or tumor origins were analyzed by the Student's or

Welch's t-test. A value of $P < 0.05$ was considered to indicate a statistically significant result.

Results

TRB3 expression in the M2TRB3 cells. To examine the role of TRB3 in cell proliferation, we developed a cell line (M2TRB3) that stably expresses the human TRB3 gene by transfecting the gene into murine mammary tumor cell line Cl66M2 (M2) (18). We also developed the control cells (M2mock) transfected with empty vector pcDNA3.1-Hygro. M2TRB3 cells expressed both human TRB3 mRNA and mouse TRB3 mRNA (Fig. 1A). Human embryonic kidney cell line HEK293 and human hepatoma cell line HepG2 also expressed human TRB3 mRNA. There was no mRNA expression of human TRB3 in the M2 and M2mock cells. Human TRB3 DNA was PCR-amplified from pcDNA3.1-Hygro-flag-human TRB3 vector and the band was present in the positive lane in Fig. 1A. Expression of the exogenous TRB3 protein (Flag) was present in the M2TRB3 cells. No expression was noted in the protein samples derived from M2, M2mock, HEK293 and HepG2 cells (Fig. 1B). The M2TRB3 and M2mock cells were used for cell proliferation assays.

TRB3 gene enhances cell proliferation and tumor volume. To examine the tumorigenic activity of the TRB3 gene, we investigated its effects on cell and tumor growth using M2TRB3 and M2mock cells. The number of M2TRB3 cells significantly increased compared to M2mock cells at the 48 and 72 h time points ($P < 0.01$ and 0.05, respectively) (Fig. 2A). At 72 h, a 34% increase was noted in the number of M2TRB3 cells compared to that of the M2mock cells. To examine the extent of M2TRB3 cell growth when these cells were implanted into the subcutaneous tissue of mice, we used the xenograft mouse model as described in Materials and methods. Twice a week observation was carried out with all mice throughout the experiment. Tumor growth was monitored with the naked eye from day 7 to 35 after inoculation; tumor volume (mm^3) and body weight (g) were measured twice weekly. No specific physical and behavioral changes were noted in any mice. The average volume of the tumors derived from the M2TRB3 cells was significantly increased by 38% when compared with that of the M2mock tumors at experimental day 35 ($P < 0.05$) (Fig. 2B).

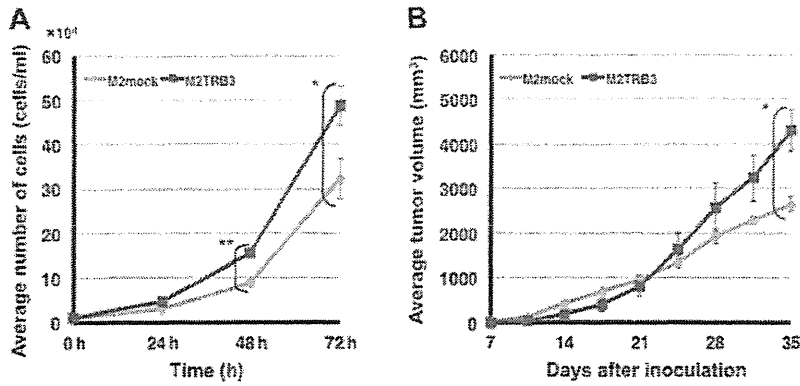


Figure 2. Growth enhancing effects of the TRB3 gene. (A) Growth curve of the cell proliferation assay. Asterisk(s) indicate statistical significance between the two cell lines (* $P < 0.05$, ** $P < 0.01$). (B) Volume of the M2TRB3 tumors and M2mock tumors. Asterisk indicates statistical significance in tumor volume between the two tumors (* $P < 0.05$).

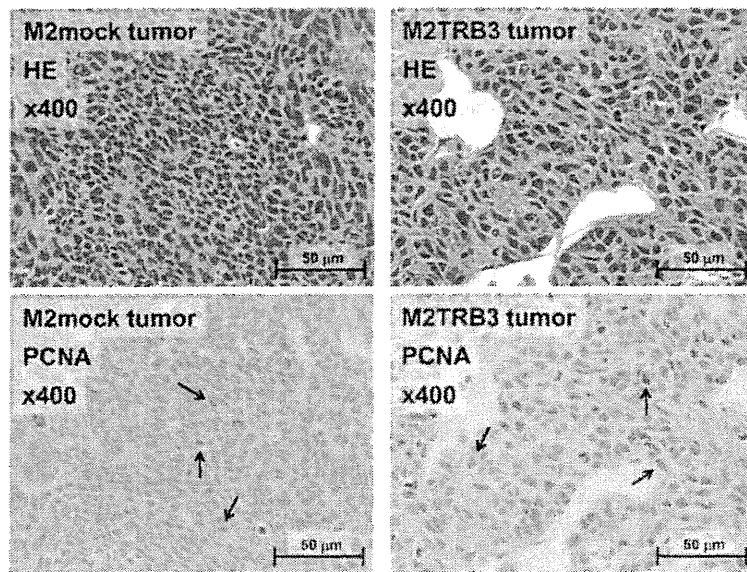


Figure 3. Representative histological features of the M2mock (left rows) and M2TRB3 tumors (right rows). Upper panels were stained with hematoxylin and eosin (HE) and lower panels show immunohistochemical staining of proliferating cell nuclear antigen (PCNA). Approximately 20 and 35% of M2mock and M2TRB3 cells were PCNA-positive (arrows), respectively. Magnification was $\times 400$ in all four images.

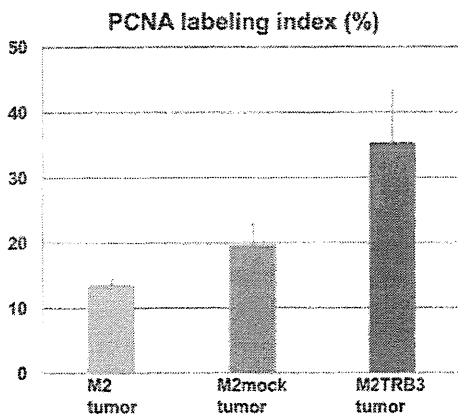


Figure 4. PCNA labeling index in the M2, M2mock and M2TRB tumors. A marked difference was noted between M2mock and M2TRB3 tumors but this difference did not achieve statistical significance.

Gross and histological features and PCNA labeling index of the M2TRB3 tumors. Due to the growth enhancing effects identified above, we aimed to determine whether the TRB3 gene induced morphological changes in tumor tissues. The M2TRB3, M2mock and M2 tumors were excised from the mouse skin, fixed with 10% buffered formalin, and stained with hematoxylin and eosin for histological examination. The tumors were analyzed using a light microscope. M2TRB3 and M2mock tumors presented a pedunculated round shape. Histologically, M2mock tumor cells grew in a solid appearance (Fig. 3, left upper panel). A site of necrosis was present in the central region of the tumor. M2TRB3 tumors were also solid and papillary growth was partially noted (Fig. 3, right upper panel). In the M2TRB3 tumors, the mean diameter of the nucleus ($9.4 \pm 0.3 \mu\text{m}$) was significantly greater than that ($7.0 \pm 0.2 \mu\text{m}$) of the M2mock tumors ($P < 0.001$). The cell proliferation rate was evaluated by measuring the PCNA

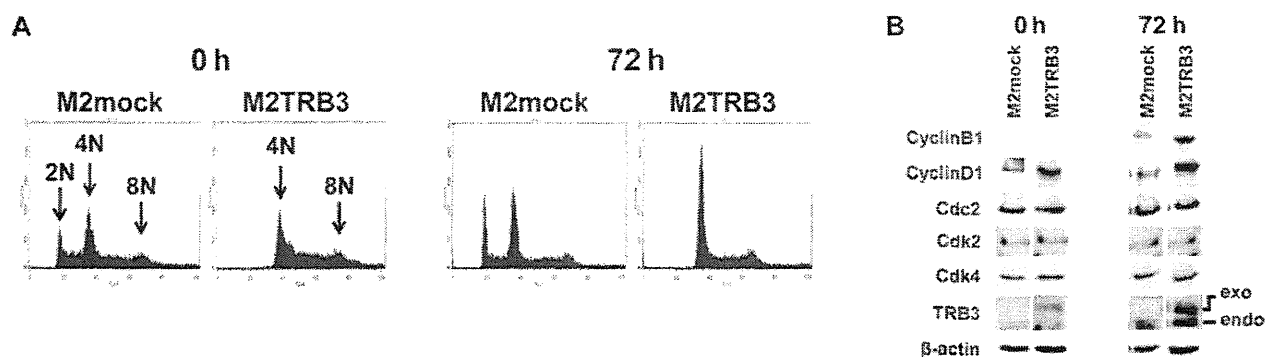


Figure 5. Effects of the TRB3 gene on DNA ploidy and protein expression. (A) Representative results of the flow cytometric analysis. After synchronizing cells in the G0/G1 phase, ploidy status was determined by flow cytometric analysis at 0 (left two panels) and 72 h (right two panels) in the M2mock and M2TRB3 cell lines. Note three peaks (2N, diploid; 4N, tetraploid; 8N, octaploid) in different locations in the DNA histogram. (B) Protein expression status of cell cycle control molecules and TRB3 in M2mock and M2TRB3 cell lines. Cell lysates were extracted at the indicated times (h) of the cell culture and then examined by western blot analysis for the indicated proteins, using the respective antibodies, as described in Materials and methods. β -actin was used as an internal control. Exogenous human TRB3 and endogenous mouse TRB3 are describes as exo and endo, respectively.

Table I. Distribution and rate (%) of ploidy in the M2TRB3 and M2mock cell lines.

Cell line	Ploidy	Time (h) after starvation	
		0 h	72 h
M2mock	2N	12.1 \pm 0.3 ^a	16.6 \pm 0.1 ^b
M2TRB3	2N	0.0 \pm 0.0	0.0 \pm 0.0
M2mock	4N	37.0 \pm 0.1	38.5 \pm 0.2 ^b
M2TRB3	4N	41.6 \pm 2.5	57.5 \pm 0.5
M2mock	8N	6.0 \pm 0.1 ^a	3.5 \pm 0.0 ^b
M2TRB3	8N	25.3 \pm 1.8	16.2 \pm 0.3

Differences in the percentage of ploidy in M2TRB3 and M2mock cell lines. M2TRB3 vs. M2mock, ^aP<0.01 and ^bP<0.001.

labeling index as described in Materials and methods. The PCNA labeling index of the M2TRB3 tumors was higher than that of the M2 and M2mock tumors but this difference was not statistically significant (Fig. 3 lower panels and Fig. 4).

TRB3 affects the ploidy distribution of mouse mammary tumor cells. Due to the differences in tumor morphology noted in Fig. 3, we examined the effects of the TRB3 gene on DNA ploidy in M2mock and M2TRB3 cells. After synchronizing cells in the G0/G1 phase, we conducted experiments at 0 and 72 h using flow cytometric analysis. Representative DNA histograms of the analysis for these cells are shown in Fig. 5A, and distribution and rate of DNA ploidy are shown in Table I. In M2mock cells, the average percentage of diploid nuclei measured 12-16%. In contrast, no diploid nuclei were observed in M2TRB3 cells (Table I and Fig. 5A, far right panels). M2TRB3 cells showed a significant increase by 19 and 12% in the population of octaploid nuclei at 0 and 72 h, respectively, when compared to M2mock cells (Table I). There was also an increase (4-19%) in the population of tetraploid nuclei in the M2TRB3 cells. These results indicate that TRB3

affects the status of DNA ploidy in mouse mammary tumor cells. M2mock cells exhibited population peaks of aneuploid nuclei (2N, 4N and 8N), indicating that these cells harbor a variable number of chromosomes.

Expression status of TRB3 and cell cycle control molecules in M2TRB3 and M2mock cells. Due to the growth enhancing effects of TRB3 as noted in Fig. 2 and nuclear hyperploidy in M2TRB3 cells, we examined whether these cells affected the levels of expression of TRB3 and cell cycle control molecules. Thus, we measured the protein expression levels of TRB3 and cell cycle control molecules cyclin B1, cyclin D1, Cdc2, Cdk2 and Cdk4. In M2TRB3 cells, both exogenous and endogenous TRB3 were highly expressed at 72 h compared to M2mock cells that only expressed endogenous TRB3 (Fig. 5B, right two columns). In contrast, a weak expression level of endogenous TRB3 was observed at 0 h in the M2mock cells, and marginal expression was noted in both exogenous and endogenous TRB3 at the same time point in these cells. Cyclin B1 and cyclin D1 expression levels in the M2TRB3 cells increased at 72 h of the cell culture, in which tumor cells were out of synchrony, compared to those of the M2mock cells (Fig. 5B). Expression levels of Cdc2, Cdk2, and Cdk4 showed no change between the M2TRB3 and M2mock cells.

Discussion

Several human tumor tissues have recently been shown to highly express TRB3 mRNA (14). It has also been demonstrated by us that TRB3 regulates the stability of Cdc25A, an essential activator of CDKs (10). However, the precise role and functional morphology of TRB3 have not been established yet. Thus, we carried out the present study to provide further evidence concerning cell growth and morphological changes in mouse mammary tumor cells by focusing on the expression levels of TRB3 and cell cycle control molecules, cellular nucleus size, and the status of DNA ploidy. M2TRB3 cells showed a significant numerical increase compared to the control M2mock cells. As a result, the doubling time of the M2TRB3 and M2mock cell lines was approximately 12

and 15 h, respectively (Fig. 2A). A similar condition was also observed in the tumors, clearly indicating that in this context TRB3 had an enhancing property on the growth of mouse mammary tumor cells.

It is well understood that cell volume increases with DNA ploidy, and this correlation has been observed in a wide variety of eukaryotic cells (19). Increased DNA ploidy can exert its effects by increasing nuclear size, chromatin content, and the expression levels of a certain gene (19). We found that in the M2TRB3 tumors the mean diameter of the nucleus measured $9.4 \pm 0.3 \mu\text{m}$ and that of the M2mock tumors was $7.0 \pm 0.2 \mu\text{m}$. From the flow cytometric analysis we also found a significant increase in the population of M2TRB3 cells bearing tetraploid or octaploid nuclei compared to that of the M2mock cells bearing mostly diploid or tetraploid nuclei (Fig. 5A). These findings are consistent with those reported by Danielsen *et al.* (20) who demonstrated that nuclei of $6.0\text{--}7.5 \mu\text{m}$ in diameter are classified as diploid, $7.5\text{--}9.0 \mu\text{m}$ as tetraploid, and $9.5\text{--}11.0 \mu\text{m}$ as octaploid. Collectively, TRB3 may have the ability of polyploidization during development.

Cyclins are the key molecules in cell cycle control due to their specific and periodic expression during cell cycle progression. Cyclin D1 complexes with Cdk4 and Cdk6 and thereby regulates transition from the G1 phase into the S phase by phosphorylation and inactivation of pRB (21-24). Phosphorylation causes release of the transcription factor E2F that promotes mitosis (24,25). Gene amplification and/or protein overexpression of cyclin D1 occurs in a variety of human carcinomas and tumors in animal models (26,27). Unlike cyclin D1, the activity of cyclin B1 is essential for G2/M phase of the cell cycle through a complex with Cdc2 (28). However, little is known about the association between DNA ploidy and cyclin B1/cyclin D1 expression status. We found elevated expression levels of cyclin B1 and cyclin D1 in M2TRB3 cells without significant changes in expression levels of Cdc2, Cdk2 and Cdk4 (Fig. 5B). Furthermore, M2TRB3 cells totally lack diploid nuclei but a population of the M2mock cells consisted mainly of diploid or tetraploid nuclei, suggesting that expression of cyclin B1 and cyclin D1 may positively correlate with the generation of hyperploid nuclei and thereby further promote the chromosomal instability in TRB3-overexpressing cells. Similar results regarding cyclin B1/D1 overexpression and promotion of tetraploidy or aneuploidy ($>2N$) were previously obtained in human breast carcinoma and mouse myeloid cells (28,29). As we found in the present study, the novel aspect of the TRB3 gene is that this gene induces an increase in cell proliferation and polyploidy leading to enlargement of the nuclear size of the implanted mouse mammary tumor cells. These effects of TRB3 may cause chromosomal instability. The detailed mechanism of this chromosomal instability is not known but may be related to the above-described effects of TRB3 on morphological function. In a recent study, we demonstrated that TRB3 may regulate the activity of anaphase-promoting complex/cyclosome (APC/C^{dh1}) that is a major ubiquitin ligase complex regulating the progression of the cell cycle through the ubiquitination and subsequent degradation of cell cycle control molecules including cyclin B1 (30,31). In the present study, we found an elevated expression level of the cyclin B1 protein in M2TRB3 cells that overexpressed the human TRB3 gene. We should emphasize that two cell

lines M2TRB3 and M2mock differ in synchrony status that may influence their response to morphological function. This intriguing respect may also reflect the role of cell cycle progression of TRB3. Thus, it is of interest to examine whether the TRB3 gene causes *de novo* morphological changes leading to tumorigenesis in a specific organ site. An additional study using the TRB3 transgenic animal model is currently in progress to answer this question.

Acknowledgements

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Metabolomic and transcriptomic profiling of human *K-ras* oncogene transgenic rats with pancreatic ductal adenocarcinomasSetsuko Yabushita^{1,*}, Katsumi Fukamachi²,
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Pancreatic ductal adenocarcinoma (PDAC) is one of the most debilitating malignancies in humans, and one of the reasons for this is the inability to diagnose this disease early in its development. To search for biomarkers that can be used for early diagnosis of PDAC, we established a rat model of human PDAC in which expression of a human *K-ras*^{G12V} oncogene and induction of PDAC are regulated by the *Cre/lox* system. In the present study, transgenic rats bearing PDAC and control transgenic rats with normal pancreatic tissues were used for metabolomic analysis of serum and pancreatic tissue by non-targeted and targeted gas chromatography–mass spectrometry and transcriptomic analysis of pancreatic tissue by microarray. Comparison of the metabolic profiles of the serum and pancreatic tissue of PDAC-bearing and control rats identified palmitoleic acid as a metabolite, which was significantly decreased in the serum of PDAC-bearing animals. Transcriptomic analysis indicated that several transcripts involved in anaerobic glycolysis and nucleotide degradation were increased and transcripts involved in the trichloroacetic acid cycle were decreased. Other transcripts that were changed in PDAC-bearing rats were adenosine triphosphate citrate lyase (decreased: fatty acid biosynthesis), fatty acid synthase (increased: fatty acid biosynthesis) and arachidonate 5-lipoxygenase activating protein (increased: arachidonic acid metabolism). Overall, our results suggest that the decreased serum levels of palmitoleic acid in rats with PDAC was likely due to its decrease in pancreatic tissue and that palmitoleic acid should be investigated in human samples to assess its diagnostic significance as a serum biomarker for human PDAC.

Introduction

Pancreatic cancer is diagnosed in ~1 person per 10 000 annually in the USA and is the fifth leading cause of cancer mortality. Most patients die within 1 year of diagnosis, and the 5 year survival rate, less than 5%, is dismal (1). Pancreatic ductal adenocarcinomas (PDACs) are diagnosed in ≥95% of the patients with pancreatic cancer. The most widely used marker for pancreatic cancer, CA19-9, lacks specificity and sensitivity: it is elevated in cases of benign cholangitis and pancreatitis and also in other types of cancer, and it is not elevated during the early stages of PDAC, when the lesion is potentially curable (2–4).

Abbreviations: Acl, adenosine triphosphate citrate lyase; Alox5ap, arachidonate 5-lipoxygenase activating protein; Fasn, fatty acid synthase; GC-MS, gas chromatography–mass spectrometry; PCA, principal component analysis; PDAC, pancreatic ductal adenocarcinoma; TCA, trichloroacetic acid; TIC, total ion current.

Thus, there are presently no reliable biomarkers for the early detection of pancreatic cancer. Consequently, there is an urgent need for specific and sensitive biomarkers of pancreatic cancer.

Previously, we established transgenic rats in which expression of the human *H-ras*^{G12V} or *K-ras*^{G12V} oncogene is regulated by the *Cre/lox* system (5). Targeted activation of *H-ras*^{G12V} or *K-ras*^{G12V} is accomplished by injection of a *Cre*-carrying adenovirus into the pancreatic ducts through the common bile duct. Several weeks after injection, proliferative lesions in pancreatic duct epithelium, intercalated ducts and centroacinar cells, but not acinar cells, become widespread. The histopathological appearance of these adenocarcinomas closely resembles that described for typical pancreatic tumors in man. Thus, transgenic rats with induced PDACs in pancreatic tissues are an appropriate model for human PDAC. In this study, *K-ras*^{G12V} transgenic rats (*K-ras*^{G12V} Tg rats) were used because *K-ras* mutations are commonly observed in human PDAC.

Serum/plasma biomarkers are ideal in that the collection of samples is relatively non-invasive, and samples can be obtained repeatedly to monitor disease progression. Molecular profiling approaches such as transcriptomics, proteomics and metabolomics to monitor pathological processes of disease have received a great deal of attention. Metabolomic analysis refers to the comprehensive study of the many metabolites present in biological samples (6,7). The analytical techniques typically applied are nuclear magnetic resonance (8) and mass spectrometry (9), with the latter being coupled to chromatographic separation techniques such as gas chromatography or liquid chromatography. Metabolomics is a powerful method for screening biomarkers, interpreting biological pathways and understanding functions of complex biological systems (10). Recently, a urinary biomarker of prostate cancer aggressiveness was found using metabolomic profiling (11).

In the present study, we characterized the metabolite profiles of both serum and pancreatic tissue samples from control and PDAC-bearing *K-ras*^{G12V} Tg rats by non-targeted and targeted gas chromatography–mass spectrometry (GC-MS) analysis to identify potential serum biomarkers for the clinical diagnosis of PDAC. In addition, the metabolomic analysis was integrated with a transcriptomic analysis of pancreatic tissues to obtain a better understanding of the behavior of metabolites in PDAC. We identified palmitoleic acid as a metabolite that was significantly decreased in the serum of rats with PDAC, and integrated metabolomic and transcriptomic analyses suggest fluctuations in several metabolomic pathways in PDAC lesions.

Materials and methods

Ethics statement

All experiments were conducted in accordance with the 'Guidelines for Animal Experiments of Nagoya City University Graduate School of Medical Sciences', and the experimental protocol was approved by the Nagoya City University Medical School Institutional Animal Care and Use Committee.

Induction of pancreatic ductal adenocarcinoma

Kras301 rats conditionally expressing human *K-ras*^{G12V} were generated as described previously (12). An adenovirus vector carrying the *Cre* gene (AxCANCre) and an empty adenovirus vector (AxCawt) were injected into the pancreatic ducts of 10–11-week-old adult homozygous male Kras301 rats through the common duct as described previously (5,12). Six rats were treated with AxCANCre (PDAC rats) and four with AxCawt (as controls). All rats were maintained in plastic cages in an air-conditioned room with a 12 h light/12 h dark cycle and euthanized by blood withdrawal from the abdominal aorta under anesthesia 16–17 days after the injection of adenovirus vector.

Collection of pancreatic tissue and serum

Rats were killed by exsanguination under ether anesthesia. Immediately after exsanguination, pancreatic tissues were excised from euthanized rats, cut into portions and the portions either frozen in liquid nitrogen or processed for

pathological examination (see below). Collected blood samples were allowed to clot at room temperature and then centrifuged at 3000 r.p.m. for 10 min at 4°C to obtain serum. The frozen pancreatic tissues and the serum were stored in liquid nitrogen and at -80°C, respectively, until metabolomic analysis.

Pathological examination

The remaining pancreatic tissues from PDAC and control rats were fixed in 4% paraformaldehyde, processed for embedding in paraffin, cut into 3 µm sections and stained with hematoxylin and eosin for microscopic examination.

RNA extraction from pancreatic tissues

Extraction of total RNA was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantification was performed with an Amersham Pharmacia spectrophotometer, model Ultraspec 3100pro (Amersham Pharmacia Biotech, Uppsala, Sweden), and assessment of ribosomal RNA integrity was performed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA samples were stored at -80°C immediately after extraction.

Gene expression analysis

Gene expression levels were measured using a GeneChip System (Affymetrix, Santa Clara, CA). Reverse transcription of the extracted total RNA, second-strand synthesis and probe generation were all accomplished with One-Cycle Target Labeling and Control Reagents (Affymetrix) following the manufacturer's protocol. Briefly, from 5 µg of total RNA, first-strand cDNA was synthesized with SuperScript II reverse transcriptase and a T7-oligo (dT) primer, and double-strand cDNA was synthesized with *Escherichia coli* RNase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase. Biotin-labeled cRNA was prepared from the double-strand cDNA, and 15 µg of labeled cRNA was fragmented. Rat Genome 230 2.0 arrays were hybridized as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix). The arrays were stained and washed with R-Phycoerythrin Streptavidin (Molecular Probes, Eugene, OR) and with the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Fluorescence was intensified by the antibody-amplification method. The arrays were scanned with a GeneChip Scanner 3000 (Affymetrix) with Affymetrix GeneChip Command Console (AGCC) and the image files were analyzed with an Affymetrix data suite system, Expression Console (EC) version 1.1; the tab-delimited files obtained contained data regarding the relative levels of expression of transcripts (signal) and the reliability of detection (Detection Call). The derived signal values were globally normalized to 100.

Sample preparation for GC-MS analysis

Pancreatic tissue and serum were subjected to two derivatization steps for GC-MS analysis. Pancreatic tissue samples (45–84 mg) were weighed and homogenized in a 12-fold volume solution of methanol/water/10 mM 2-hydroxyundecanoic acid (800:100:1, v/v/v; 10 mM 2-hydroxyundecanoic acid was used as an internal standard) using a potter homogenizer on ice, followed by centrifugation at 15 000 r.p.m. for 10 min at 4°C. Serum samples (50 µl) were deproteinized by addition of an 8-fold volume solution of methanol/water/10 mM 2-hydroxyundecanoic acid (800:100:1, v/v/v; 10 mM 2-hydroxyundecanoic acid was used as an internal standard), followed by centrifugation at 15 000 r.p.m. for 5 min at 4°C. The supernatant (200 µl) was dried in a vacuum freeze dryer, 30 µl of methoxyamine hydrochloride (Sigma-Aldrich) in pyridine (10 mg/ml) was added as a derivatizing agent and the mixture was incubated at 30°C for 90 min. Thereafter, 30 µl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (GL Science, Tokyo, Japan) was added for derivatization, and the mixture was incubated at 40°C for 30 min.

GC-MS analysis

All analyses were carried out with a Bruker Daltonics 1200 GC/MS/MS system (Bruker Daltonics K.K., Japan) using an Agilent Technologies VF5-ms capillary column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 µm). The injection temperature was 230°C. The helium gas flow rate through the column was 1 ml/min. The column oven temperature was 60°C for 2 min and rose at 10°C/min to 300°C and then remained at 300°C for 5 min. Ions were generated at an electron impact energy of 70 kV and were recorded over the mass range from *m/z*: 70 to 800.

Analysis of GC-MS data

GC-MS data were imported into LineUp (Infometrix, Bothell, WA) and PiroTrans (GL Science) in order to align the chromatogram on the basis of peak intensity and the retention time of the internal standard, 2-hydroxyundecanoic acid. The generated peak lists were imported into Pirouette software (Infometrix, Woodinville, WA) for multivariate statistical analysis. Principal component analysis (PCA) was conducted to find discriminatory metabolites in serum and pancreatic tissues of PDAC rats. Identification of discriminatory metabolites detected by PCA analysis was performed using the NIST 05 Mass Spectral Library (NIST) containing >200 000 electron impact spectra.

In addition to the automatic library search assessment on the basis of similarity score (>65), the similarity of spectra was manually confirmed.

Identification and quantification of palmitoleic acid in pancreatic tissues

To authenticate the identity of the metabolite putatively identified as palmitoleic acid, pancreatic tissues were analyzed by cochromatography with palmitoleic acid and palmitoleic acid standards (MP Biomedicals, CA). The identity of the metabolite was determined based on comparison of the retention time and fragment pattern of the metabolite and those of the two standards.

For concentration measurements, palmitoleic acid was diluted in pyridine and six standards of various mixtures of compounds of known concentration between 0.05 and 100 p.p.m. were prepared. The concentration of palmitoleic acid in the samples was quantified by a standard curve of total ion current (TIC) chromatograms or *m/z* 311 mass chromatograms.

Statistics

The peak intensities of metabolites and the signal values of gene expression were statistically analyzed by the Student's *t*-test between PDAC and control rats, with a level of probability of 0.05 used as the criterion for significance.

Results

Gross and histological observation

Kras301 rats, 13–14 weeks of age, were killed 16–17 days after injection of adenovirus vector into the pancreatic duct via the common bile duct. Gross and histological findings were the same as reported previously (5, 12, 13). PDAC was well developed with grossly visible whitish lesions in all six rats treated with AxCANCre. Histopathological examination showed that the tumors were adenocarcinomas with a variable amount of fibrotic tissue, some showing desmoplastic morphology and infiltration of inflammatory cells. Invasion of the surrounding normal pancreatic tissue by the PDAC that developed in Kras301 rats was a common feature of these lesions. Figure 1 shows typical lesions that developed in the Kras301 rats.

PDAC that develop in Kras301 rats are not metastatic, and neoplastic lesions were not found in any other organs. No abnormalities were observed in pancreatic tissues of the four rats injected with AxCAwt.

GC-MS chromatograms of serum and pancreatic tissues

Typical GC-MS TIC chromatograms of serum and pancreatic tissue extracts are shown in Supplementary Figure 1, available at *Carcinogenesis* Online. The retention time of the internal standard was consistent for each run. The data showed marked differences in the chromatogram patterns between the serum and pancreatic tissue.

Clustering of control and PDAC samples

Score plots produced by PCA of the serum pancreatic tissue are shown in Figure 2. The PCA algorithm generates a single point that represents the metabolites in a sample and their concentrations (each dot in the score plots shown in Figure 2 represents a single rat): close clustering of dots indicates that the samples have similar compositions. The control and PDAC samples clustered into two distinct groups in both the serum (Figure 2A) and pancreatic tissue (Figure 2B) score plots, indicating that the profiles of the metabolites in the serum and pancreatic tissue samples derived from the control rats differed from those derived from the rats with PDAC.

Metabolite differences between control and PDAC samples screened by PCA

In addition to the score plot, which is based on the composition of the samples, the PCA algorithm also creates a loading plot, which is based on metabolite values. Loading plots identify metabolites that contribute to the differential clustering of PDAC and control samples in the score plots. The electron impact spectra of these discriminatory metabolites were then compared with the NIST 05 Mass Spectral Library (NIST). Putatively identified discriminatory metabolites with matching NIST spectra are shown in Table 1. Fold changes and *P*-values of these metabolites in the serum and pancreatic tissues were calculated by the TICs of GC-MS chromatograms.

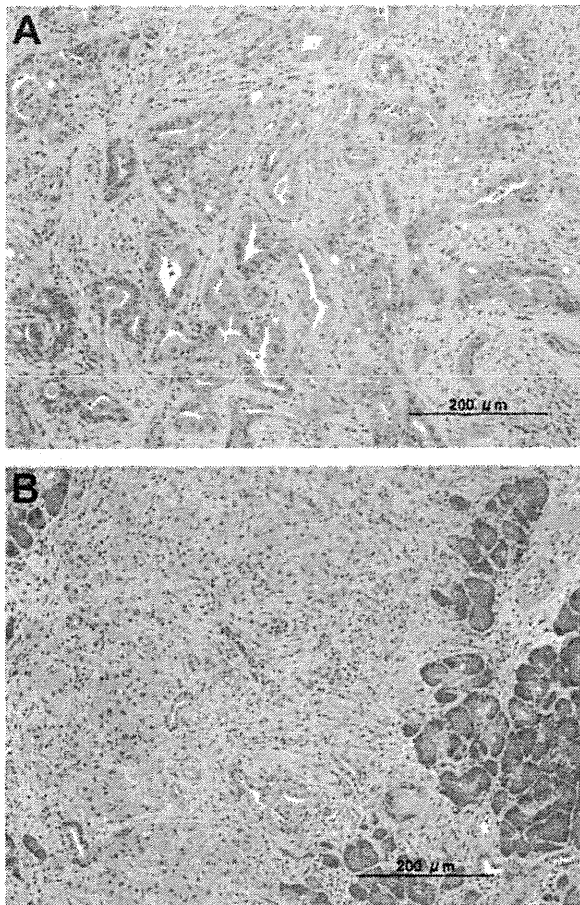


Fig. 1. Typical lesions that developed in the Kras301 rats are shown. (A) An adenocarcinoma with desmoplastic appearance. (B) An adenocarcinoma invading the surrounding pancreas tissue.

In the serum, aminomalonic acid, 2-aminoethyl dihydrogen phosphate, citrate and lanthionine were significantly increased and chenodeoxycholic acid was non-significantly increased (1.9-fold). In pancreatic tissues, however, none of these metabolites was significantly increased and aminomalonic acid was significantly decreased.

A number of fatty acids were decreased in pancreatic tissues: palmitoleic acid, linoleic acid, octadecanoic acid, myristic acid, arachidonic acid and hexadecanoic acid were significantly decreased and tridecanoic acid, 12-methyl-, methyl ester and heptadecanoic acid were non-significantly decreased. Of these fatty acids, only palmitoleic acid (significantly decreased) and arachidonic acid (non-significantly decreased) were also decreased in the serum.

A third metabolite that had corresponding fluctuations in both the serum and pancreatic tissue was 2(1*H*)-pyrimidinone. This metabolite was significantly decreased in pancreatic tissues and non-significantly in the serum.

None of the other discriminatory metabolites had corresponding fluctuations in both the serum and pancreatic tissue. Beta-alanine, xanthine and uridine were increased in pancreatic tissue but not in the serum, and aspartic acid, alpha-glycerophosphoric acid, tyrosine, 2-monopalmitin, adenosine monophosphate and purine were decreased in pancreatic tissue but not in the serum.

Overall, the metabolic profiles in the pancreatic tissue and the serum of control and PDAC-bearing rats were different, with several discriminatory metabolites contributing to this difference. The array of changes in the individual discriminatory metabolites in the serum and pancreatic tissue, however, were distinct.

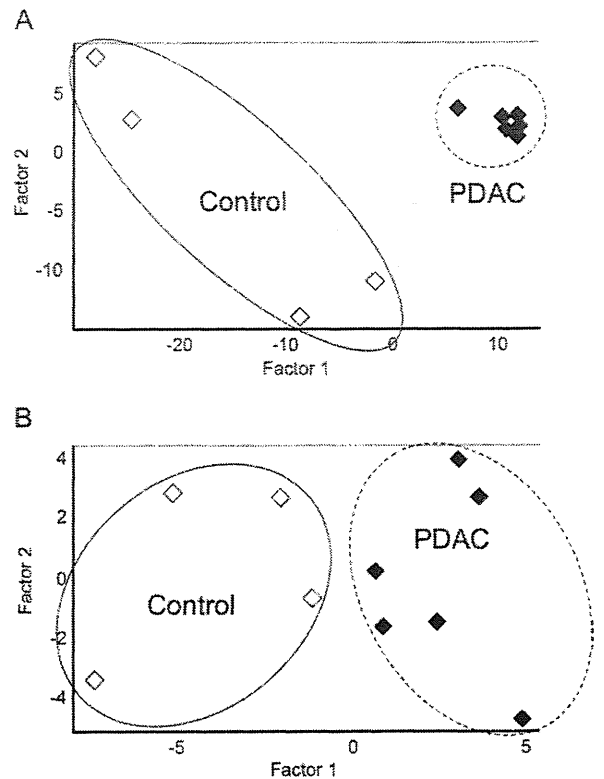


Fig. 2. Score plots of PCA based on the metabolite profile data of PDAC rats and control rats. The rhombus plots (filled and open) indicate the results for PDAC rats ($n = 6$) and control rats ($n = 4$), respectively. (A) Score plots of PCA based on the metabolite profile data for pancreatic tissues discriminating between PDAC and control rats. The principal components PC1 ($t[1]$) and PC2 ($t[2]$) described 59.6 and 14.9% of the variation. (B) Score plots of PCA based on the metabolite profile data of serum discriminating between PDAC and control rats. The principal components PC1 ($t[1]$) and PC2 ($t[2]$) described 32.8 and 18.0% of the variation.

Identification and quantification of palmitoleic acid in pancreatic tissue and serum using standard chemicals

Once a metabolite that was significantly decreased in both pancreatic tissues and serum was detected and putatively identified as palmitoleic acid, the identification of the metabolite was authenticated by cochromatography with palmitoleic acid and palmitoleic acid. The retention time and fragment pattern of the standards identified the putatively annotated palmitoleic acid as 'palmitoleic acid'.

A standard curve was then used for concentration measurement in samples: the linear region of quantitation was between 0.1 and 100 p.p.m. ($R^2 = 0.9857$). The concentrations of palmitoleic acid in pancreatic tissues and serum of the individual control and PDAC-bearing rats are shown in Table II. The palmitoleic acid concentration in the pancreatic tissues of control and PDAC rats was 266.5 186.2 and 7.5 4.6 p.p.m., respectively, and the concentration of palmitoleic acid in the serum samples of control and PDAC-bearing rats was 2.6 0.8 and 1.2 0.1 p.p.m., respectively (P -value: 0.031).

Alterations in the levels of metabolites involved in glycolysis and the trichloroacetic acid cycle

The 'Warburg effect' in which tumor cells rapidly use glucose and convert the majority of it to lactate is known as a typical signature of cancer (14,15). Since we observed decreases for a number of fatty acids, which are functionally related to the glycolytic pathway, in the pancreatic tissue of PDAC-bearing rats, we searched for peaks

Table I. Fold changes of the discriminatory metabolites characterizing PDAC tissues and serum

Metabolites	Retention time (min)	Serum		Pancreatic tissues	
		PDAC/control		PDAC/control	
		Fold changes	P-values	Fold changes	P-values
Threonine	10.37	1.2 ^a	0.373	1.3	0.330
Beta-alanine	10.94	ND		1.7 ^a	0.072
Aminomalonic acid	11.46	1.3	0.011*	0.8 ^a	0.019*
Aspartic acid	12.07	1.3	0.106	0.7 ^a	0.005*
Tridecanoic acid, 12-methyl-, methyl ester	14.53	ND		0.1 ^a	0.051
Alpha-glycerophosphoric acid	14.79	1.4	0.060	0.8 ^a	0.000*
2-Aminoethyl dihydrogen phosphate	15.04	1.2 ^a	0.035*	1.0	0.257
Citrate	15.40	1.3 ^a	0.017*	1.2	0.522
Tyrosine	16.67	1.2	0.086	0.3 ^a	0.024*
Xanthine	17.45	1.1	0.405	1.7 ^a	0.016*
Palmitelaidic acid	17.54	0.6 ^a	0.002*	0.1 ^a	0.001
Heptadecanoic acid	18.62	0.9	0.277	0.3 ^a	0.062
Linoleic acid	19.24	0.9	0.683	0.2 ^a	0.000*
Octadecanoic acid	19.57	ND		0.7 ^a	0.001*
Lanthionine	19.87	1.4 ^a	0.027*	1.0	0.949
Myristic acid	20.46	0.9	0.138	0.1 ^a	0.020*
Arachidonic acid	20.56	0.4 ^a	0.098	0.6 ^a	0.021*
Uridine	21.22	ND		3.2 ^a	0.001*
2-Monopalmitin	21.97	1.0	0.205	0.05 ^a	0.001*
Hexadecanoic acid	22.22	0.9	0.203	0.1 ^a	0.038*
2-(1H)-Pyrimidinone	23.96	0.5	0.316	0.5 ^a	0.000*
Adenosine monophosphate	25.55	0.9	0.304	0.5 ^a	0.000*
Purine	25.85	ND		0.4 ^a	0.000*
Chenodeoxycholic acid	27.14	1.9 ^a	0.148	1.1	0.643

Sera and pancreatic tissues from PDAC rats ($n = 6$) and controls ($n = 4$) were analyzed. ND, peaks were not detected.

^aMetabolites that contributed to the differentiation of PDAC and control samples in the PCA analysis.

* $P < 0.05$.

Table II. Concentration of palmitoleic acid in the tissue and serum of control and PDAC rats

Rat no.	Concentration in tissue or serum (p.p.m. ^a)		t-Test
Pancreas control			
30	347.72	266.5 ± 186.2	
38	490.90		
50	127.08		
83	100.33		
Pancreas PDAC			
42	10.58	7.5 ± 4.6	0.069
47	2.01		
84	3.78		
86	13.77		
87	4.97		
88	10.18		
Serum control			
30	2.77	2.6 ± 0.8	
38	3.65		
50	1.97		
83	2.10		
Serum PDAC			
42	1.15	1.2 ± 0.1	0.031
47	0.96		
84	1.34		
86	1.28		
87	1.09		
88	1.20		

^ap.p.m. in tissue is microgram palmitoleic acid per gram tissue and p.p.m. in serum is microgram palmitoleic acid per milliliter serum.

of metabolites involved in glycolysis and the trichloroacetic acid (TCA) cycle. Glucose, pyruvate, lactate, succinate, fumarate, malate and alpha-keto-glutarate were identified. The changes in the levels of these metabolites in the serum and pancreatic tissues were calculated

by the TICs of GC-MS chromatograms (Table III). No significant differences between PDAC-bearing rats and control rats were found.

Transcriptional changes related to glycolysis, TCA cycle, fatty acid biosynthesis, arachidonic acid metabolism and nucleotide degradation

Transcriptional data for ~31 000 genes in PDAC and control pancreatic tissue were analyzed by microarray; the genes for which significant changes ($P \leq 0.05$) occurred are shown in Supplementary Table 1, available at *Carcinogenesis* Online. Values for enzymes involved in glycolysis, the TCA cycle, fatty acid biosynthesis, arachidonic acid metabolism and nucleotide degradation are shown in Table IV. Changes in Abat, Alox5, Dpyd, Dpys, Nt5, Uox and Upb transcription were evaluated as unclear as their expression levels were too low to be analyzed accurately by microarray analysis.

Pathways generated by integrating the results we obtained from our metabolic and transcriptional analyses are shown in Figure 3. In PDAC lesions, numerous transcripts involved in anaerobic glycolysis were upregulated and most of those involved in the TCA cycle were downregulated (Figure 3A). Transcription of two lipogenic enzymes was also changed in PDAC lesions: adenosine triphosphate citrate lyase (Acly) was decreased and fatty acid synthase (Fasn) was increased (Figure 3A).

Transcripts of arachidonate 5-lipoxygenase activating protein (Alox5ap), which is necessary for activation of Alox5, were increased (Figure 3B). This increase may be associated with the decrease of linoleic acid and arachidonic acid found in the pancreatic tissue of PDAC-bearing rats (Table I).

A number of transcripts involved in purine and pyrimidine nucleotide degradation were increased; however, transcripts involved in pyrimidine nucleotide degradation downstream of beta-alanine degradation were decreased (Figure 3C). Taken together, these changes may be associated with the changes of adenosine monophosphate, xanthine, uridine and beta-alanine found in pancreatic tissue of PDAC-bearing rats.

Table III. Fold changes of the putatively identified metabolites related to glycolysis and the TCA cycle

Metabolites	Retention time (min)	Serum		Pancreatic tissues	
		PDAC/control		PDAC/control	
		Fold changes	P-values	Fold changes	P-values
Glucose	16.17	1.0	0.510	1.0	0.940
Pyruvate	5.87	1.1	0.177	1.0	0.198
Lactate	5.95	1.0	0.456	1.0	0.373
Succinate	9.46	0.6	0.065	1.1	0.693
Fumarate	9.62	1.1	0.144	ND	
Malate	11.67	0.9	0.803	0.7	0.054
Alpha-ketoglutarate	12.75	1.0	0.832	0.8	0.631

Sera and pancreatic tissues from PDAC rats ($n = 6$) and controls ($n = 4$) were analyzed. ND, not detected.

Table IV. Microarray analysis of mRNA from pancreatic tissues from control and PDAC rats

Gene symbol	Representative public ID	Gene title	Fold	P-value
Abat	U29701	4-Aminobutyrate aminotransferase	0.0	0.077 ^a
Acly	NM_016987	Adenosine triphosphate citrate lyase	0.6	0.000*
Aco2	NM_024398	Aconitase 2, mitochondrial	0.6	0.000*
Ada	NM_130399	Adenosine deaminase	3.9	0.000*
Adar	NM_031006	Adenosine deaminase, RNA-specific	0.4	0.018*
Adar	B1292196	Adenosine deaminase, RNA-specific	0.8	0.408
Adarb1	NM_012894	Adenosine deaminase, RNA-specific, B1	0.6	0.294
Adarb1	AW253867	Adenosine deaminase, RNA-specific, B1	0.1	0.066
Adarb1	AW522471	Adenosine deaminase, RNA-specific, B1	3.4	0.000*
Adarb2	NM_133302	Adenosine deaminase, RNA-specific, B2	3.3	0.012*
Adat3	AI029510	Adenosine deaminase, tRNA-specific 3. TAD3 homolog (<i>S. cerevisiae</i>)	0.4	0.139
Aldh6a1	NM_031057	Aldehyde dehydrogenase 6 family, member A1	0.2	0.001*
Aldoa	NM_012495	Aldolase A, fructose bisphosphate	4.0	0.001*
Aldob	M10149	Aldolase B, fructose bisphosphate	0.0	0.146
Aldoc	NM_012497	Aldolase C, fructose bisphosphate	0.6	0.100
Alox5	NM_012822	Arachidonate 5-lipoxygenase	2.1	0.081 ^a
Alox5ap	NM_017260	Arachidonate 5-lipoxygenase activating protein	3.0	0.000*
Ampd1	J02811	Adenosine monophosphate deaminase 1 (isoform M)	0.8	0.707
Ampd2	BE111787	Adenosine monophosphate deaminase 2 (isoform L)	0.7	0.029*
Ampd2	BE100752	Adenosine monophosphate deaminase 2 (isoform L)	1.5	0.068
Ampd3	NM_031544	Adenosine monophosphate deaminase 3	2.6	0.006*
Cda	AA859352	Cytidine deaminase	2.7	0.031*
Cs	NM_130755	Citrate synthase	1.1	0.119
Cs	AI009657	Citrate synthase	1.2	0.020*
Cs	H33235	Citrate synthase	1.3	0.013*
Dpyd	NM_031027	Dihydropyrimidine dehydrogenase	0.2	0.106 ^a
Dpys	NM_031705	Dihydropyrimidinase	0.0	0.377 ^a
Eno1	NM_012554	Enolase 1 (alpha)	3.7	0.000*
Eno2	AF019973	Enolase 2 (gamma), neuronal	5.9	0.000*
Eno3	NM_012949	Enolase 3, beta, muscle	0.7	0.457
Fasn	NM_017332	Fatty acid synthase	0.8	0.433
Fasn	AI179334	Fatty acid synthase	2.1	0.000*
Fh1	NM_017005	Fumarate hydratase 1	0.9	0.355
Gapdh	AFFX_Rat_GAPDH_3	Glyceraldehyde-3-phosphate dehydrogenase	3.0	0.001*
Gapdh	AFFX_Rat_GAPDH_5	Glyceraldehyde-3-phosphate dehydrogenase	2.7	0.003*
Gapdh	AFFX_Rat_GAPDH_M	Glyceraldehyde-3-phosphate dehydrogenase	2.6	0.003
Gapdh /// Gapdh-ps2	NM_017008	Glyceraldehyde-3-phosphate dehydrogenase /// glyceraldehyde-3-phosphate dehydrogenase, pseudogene 2	4.0	0.001*
Gek	NM_012565	Glucokinase	0.3	0.152
Gda	AF245172	Guanine deaminase	3.8	0.001*
Got1	D00252	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	1.1	0.589
Got2	NM_013177	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	2.1	0.002*
Got2 /// LOC297793 /// LOC314123	BI296539	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) /// similar to aspartate aminotransferase, mitochondrial precursor (transaminase A) (glutamate oxaloacetate transaminase 2) /// similar to aspartate aminotransferase, mitochondrial precursor (transaminase A) (glutamate oxaloacetate transaminase 2)	1.6	0.029*
Gpd1	NM_022215	Glycerol-3-phosphate dehydrogenase 1 (soluble)	1.3	0.737

Table IV. *Continued*

Gene symbol	Representative public ID	Gene title	Fold	P-value
Gpd1	BI277042	Glycerol-3-phosphate dehydrogenase 1 (soluble)	0.3	0.048*
Gpi	BI283882	Glucose phosphate isomerase	3.9	0.000*
Gpt	NM_031039	Glutamic-pyruvate transaminase (alanine aminotransferase)	0.1	0.009*
Hk1	NM_012734	Hexokinase 1	0.8	0.476
Hk1	AFFX_Rat_012734	Hexokinase 1	2.4	0.056
Hk1	AFFX_Rat_012734	Hexokinase 1	0.8	0.559
Hk1	AFFX_Rat_012734	Hexokinase 1	0.7	0.319
Hk2	NM_012735	Hexokinase 2	1.3	0.366
Hk2	BI294137	Hexokinase 2	1.8	0.001*
Hprt1	M86443	Hypoxanthine phosphoribosyltransferase 1	1.3	0.047*
Idh2	AI172491	Isocitrate dehydrogenase 2 (NADP ⁺), mitochondrial	0.5	0.000*
Idh3a	NM_053638	Isocitrate dehydrogenase 3 (NAD ⁺) alpha	2.3	0.001*
Idh3B	AI171793	Isocitrate dehydrogenase 3 (NAD ⁺) beta	0.7	0.000*
Idh3g	BI277627	Isocitrate dehydrogenase 3 (NAD), gamma	0.6	0.001*
Ldha	NM_017025	Lactate dehydrogenase A	2.6	0.004*
Ldhb	AA848319	Lactate dehydrogenase B	2.3	0.005*
Ldhc	NM_017266	Lactate dehydrogenase C	0.4	0.334
Ldhd	AI145761	Lactate dehydrogenase D	0.7	0.380
Ldhd	AI501131	Lactate dehydrogenase D	0.4	0.051
Mdh1	NM_033235	Malate dehydrogenase 1, NAD (soluble)	0.6	0.003*
Mdh1	BG671530	Malate dehydrogenase 1, NAD (soluble)	0.6	0.004*
Mdh2	NM_031151	Malate dehydrogenase 2, NAD (mitochondrial)	0.6	0.009
Me1	M30596	Malic enzyme 1, NADP(+)-dependent, cytosolic	3.1	0.003*
Me1	NM_012600	Malic enzyme 1, NADP(+)-dependent, cytosolic	2.0	0.035*
Nt5c3	AI409146	5'-Nucleotidase, cytosolic III	0.8	0.151
Nt5c3l	BF412799	5'-Nucleotidase, cytosolic III like	0.9	0.360
Nt5e	NM_021576	5'-Nucleotidase, ecto	1.1	0.745
Nt5e	BI289470	5'-Nucleotidase, ecto	1.1	0.696
Nt5m	AA819763	5',3'-Nucleotidase, mitochondrial	2.6	0.052
Ogdh	BI277513	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	1.3	0.004*
Ogdh	BE103050	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	1.0	0.968
Pc	NM_012744	Pyruvate carboxylase	0.4	0.016*
Pdha1	AI411413	Pyruvate dehydrogenase (lipoamide) alpha 1	0.7	0.005*
Pdha1	BF561717	Pyruvate dehydrogenase (lipoamide) alpha 1	0.5	0.006*
Pdha2	NM_053994	Pyruvate dehydrogenase (lipoamide) alpha 2	0.1	0.302
Pdhb	BM389223	Pyruvate dehydrogenase (lipoamide) beta	0.4	0.001*
Pfk1	NM_013190	Phosphofructokinase, liver	1.0	0.952
Pfkm	NM_031715	Phosphofructokinase, muscle	0.8	0.001*
Pfkm	AI071717	Phosphofructokinase, muscle	0.3	0.143
Pfkm	BI291434	Phosphofructokinase, muscle	0.4	0.165
Pfkm	BM389769	Phosphofructokinase, platelet	3.5	0.001*
Pgam1	NM_053290	Phosphoglycerate mutase 1 (brain)	1.8	0.004*
Pgam2	NM_017328	Phosphoglycerate mutase 2 (muscle)	0.7	0.287
Pgk1	NM_053291	Phosphoglycerate kinase 1	0.3	0.131
Pgk1	NM_053291	Phosphoglycerate kinase 1	3.4	0.005*
Pgk1	BI279760	Phosphoglycerate kinase 1	2.2	0.011*
Pkir	NM_012624	Pyruvate kinase, liver and RBC	0.1	0.207
Pkir	M17685	Pyruvate kinase, liver and RBC	0.3	0.273
Pkm2	NM_053297	Pyruvate kinase, muscle	7.7	0.001*
Sdha	NM_130428	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	0.8	0.009*
Sdhb	AI172320	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	0.9	0.099
Sdhc	AI009817	Succinate dehydrogenase complex, subunit C, integral membrane protein	0.7	0.001*
Sdhd	AI410703	Succinate dehydrogenase complex, subunit D, integral membrane protein	1.0	0.911
Sdhd	AI176608	Succinate dehydrogenase complex, subunit D, integral membrane protein	0.8	0.009*
Suca2	BF412750	Succinate-CoA ligase, ADP-forming, beta subunit	0.9	0.680
Suca2	H31112	Succinate-CoA ligase, ADP-forming, beta subunit	0.6	0.596
Suca2	AA923982	Succinate-CoA ligase, ADP-forming, beta subunit	0.7	0.001*
Suc1g1	NM_053752	Succinate-CoA ligase, alpha subunit	0.5	0.000*
Suc1g2	AI237518	Succinate-CoA ligase, GDP-forming, beta subunit	0.4	0.000*
Tpi1	NM_022922	Triosephosphate isomerase 1	3.0	0.001*
Uox	M24396	Urate oxidase	0.1	0.221 [†]

Table IV. Continued

Gene symbol	Representative public ID	Gene title	Fold	P-value
Upbl	NM_053845	Ureidopropionase, beta	0.1	0.387 ^a
Uppi	BI292558	Uridine phosphorylase 1	9.8	0.000*
Xdh	NM_017154	Xanthine dehydrogenase	2.5	0.001*

Expression levels of mRNAs related to glycolysis, the TCA cycle, fatty acid synthesis, nucleotide metabolism and the arachidonic acid cascade are shown. Pancreatic tissues derived from PDAC rats ($n = 6$) and controls ($n = 4$) were analyzed. P-values were calculated using Student's *t*-test.

^aExpression levels were too low to be analyzed accurately by microarray analysis.

* $P < 0.05$.

Discussion

Serum metabolites are ideal biomarkers of human disease as the collection of serum samples is relatively non-invasive and multiple samples can be obtained to monitor disease progression. However, since serum represents the effects of metabolism in multiple organs, it is difficult to assign a metabolic fingerprint to specific metabolic processes of disease. Despite this, metabolites that change in the serum and display a similar change in diseased tissue, such as a cancerous lesion, are potential biomarkers specific to the metabolic process of the disease.

In this study, sera and pancreatic tissues from four control and six PDAC rats were subjected to non-targeted and targeted GC-MS-based metabolomics. The PCA score plots of the non-targeted metabolomic data of both the serum and pancreatic tissue showed a clear separation between control and PDAC rats, indicating the presence of distinct metabolite profiles in the serum and pancreatic tissue of PDAC-bearing rats compared with control rats.

Palmitoleic acid, which contributed to the distinctive metabolic profiles of the samples from the PDAC-bearing and control rats, was significantly decreased in the serum. Palmitoleic acid is a monounsaturated fatty acid biosynthesized from palmitic acid. Since palmitoleic acid is also contained in the diet, the decreases in the serum and pancreatic tissue of PDAC-bearing rats could be due to decreased absorption from the intestine. However, the decrease in PDAC-bearing rat lesions was apparently more pronounced in pancreatic tissue than in the serum. If the decrease in palmitoleic acid in the pancreatic tissue was due to its decreased levels in the serum, then the decreases found in the serum and pancreatic tissue should be similar. Instead, palmitoleic acid was decreased by a factor of ~ 2 in the serum and by a factor of ~ 7 or more in pancreatic tissue: while the decrease in palmitoleic acid in the pancreatic tissue of PDAC-bearing animals was not statistically different from the controls, it is notable that the highest level of palmitoleic acid in the PDAC rats was 13.77 p.p.m., whereas the lowest level of palmitoleic acid in the control rats was 100.33 p.p.m., a 7-fold difference. This suggests that decreased palmitoleic acid in pancreatic tissue was most likely due to increased consumption of the fatty acid in the PDAC lesions and that the decrease of palmitoleic acid in the serum was likely caused by its decrease in pancreatic tissues. Therefore, palmitoleic acid in the serum is a candidate biomarker for PDAC diagnosis.

In addition to palmitoleic acid, two other metabolites showed decreases in both the serum and pancreatic tissue: Arachidonic acid and 2(1*H*)-pyrimidinone were decreased non-significantly in the serum and decreased significantly in pancreatic tissue samples. Interestingly, the decrease of arachidonic acid could have been mediated by the increased expression of Alox5ap in PDAC (see Figure 3B). Increased activation of Alox5 could be the cause of the decrease of linoleic acid and arachidonic acid in the pancreatic tissue of PDAC-bearing rats. However, further investigations are needed to ascertain whether decreased linoleic acid and arachidonic acid were due to aberrant arachidonic acid metabolism or decreased synthesis of fatty acids.

One of the hallmarks of cancer is an increase of *de novo* fatty acid synthesis (16). A wide variety of tumors and their precursor lesions undergo exacerbated *de novo* biosynthesis of fatty acids from citrate by lipogenic enzymes—Acly, acetyl-CoA carboxylase and Fasn—irrespective of the levels of circulating lipids (16). In our

study, however, we detected a decrease in the levels of several fatty acids. Possibly, decreased Acly expression (see Figure 3A) may have caused the decreases seen in the fatty acids in the pancreatic tissue of PDAC-bearing rats.

Another hallmark of cancer is the 'Warburg effect' (14,15) and decreased glucose has been reported in the serum and cancerous tissues of oral, colon and stomach cancer patients (17,18) and increased lactate has been reported in the serum of pancreatic cancer patients (19). In the present study, transcriptional analysis of PDAC tissues also suggested activation of anaerobic glycolysis and suppression of the TCA cycle. However, neither decreased glucose nor increased lactate were observed in the serum or pancreatic tissue of PDAC-bearing rats. The lack of a 'Warburg effect' in PDAC-bearing rats was probably due to the fact that after ~ 2 weeks of development, the PDACs were not advanced enough to disrupt pancreatic function and glucose control.

A notable difference between palmitoleic acid and glucose and lactic acid is that there are metabolic pathways dedicated to the regulation of glucose and lactic acid levels, but not to palmitoleic acid levels. Consequently, the tumor-mediated fluctuations in glucose and lactic acid seen in patients with more advanced tumors (17–19) may be absent in patients (and animal models) with less advanced tumors. In contrast, fluctuations of unregulated metabolites, such as palmitoleic acid, may occur in patients (and animal models) with less advanced tumors, as seen in the present study.

The increase in nucleotide degradation products xanthine, uridine and beta-alanine coupled with increased expression of several enzymes involved in nucleotide degradation (see Figure 3C) suggests that accelerated nucleotide degradation is occurring in the PDAC lesions. This accelerated nucleotide degradation may be due to the increased turnover of nucleotides in the PDAC lesions, which results from the increased nucleic acid synthesis in proliferating cells in the lesions.

Chenodeoxycholic acid, a major constituent of bile acids, was increased in the serum of PDAC-bearing rats. Metabolomic profiling of plasma of pancreas cancer patients by Urayama *et al.* (20) also showed an elevation of the bile acids taurocholic acid and tauroursodeoxycholic acid.

Concluding remarks

In summary, metabolites in the serum and pancreatic tissue of control and PDAC-bearing *K-ras*^{G12V} transgenic rats were analyzed by non-targeted and targeted GC-MS and transcripts in the PDAC lesions were analyzed by microarray. GC-MS analysis indicated that the profiles of the metabolites in the serum and pancreatic tissue samples derived from control rats differed from those derived from PDAC-bearing rats. A metabolite identified as palmitoleic acid was significantly decreased in the serum of PDAC-bearing rats and this decrease was likely due to its decrease in pancreatic tissue. Two other interesting metabolites are linoleic acid and arachidonic acid. These metabolites were significantly decreased in the pancreases of PDAC-bearing rats, possibly due to Alox5ap-mediated upregulation of Aox5 activity. Whether these metabolites could be markers of more advanced pancreas cancer remains to be determined. Finally, integration of the results of the non-targeted GC-MS analysis and transcriptomic

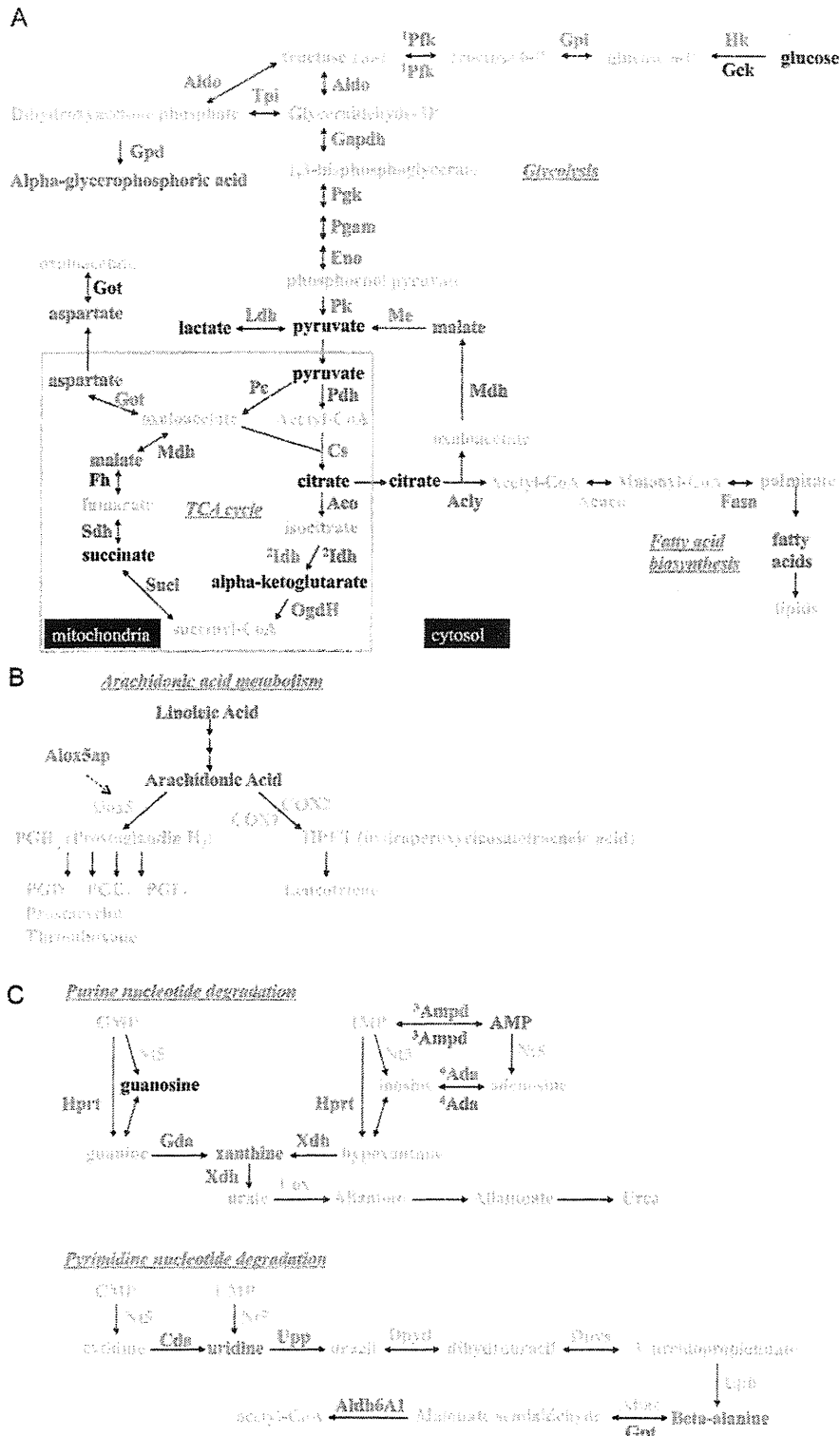


Fig. 3. Schematic representations of the most relevant metabolic and transcriptional differences in pancreatic tissues between PDAC and normal rats. Red, higher concentration in PDAC rats. Blue, lower concentration in PDAC rats. Black, not changed. Gray, not determined or unclear. ¹Pfk was increased, whereas Pfk_m was decreased. ²Idh3a was increased, whereas Idh2, Idh3B and Idh3g were decreased. ³Ampd3 was increased, whereas Ampd2 was decreased. ⁴Ada, Adarb1 and Adarb2 were increased, whereas Adar was decreased.

analysis indicated accelerated anaerobic glycolysis and nucleotide degradation and suppressed TCA cycle and fatty acid biosynthesis in the PDAC lesions.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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