Abstract. Epithelial-mesenchymal transition (EMT) is considered to serve an important role in the metastatic/invasive ability of cancer cells, in the acquisition of drug resistance, and in metabolic reprogramming. In the present study, it was hypothesized that the Klotho gene is involved in the metastatic/invasive ability of lung cancer. We previously reported an association between Klotho expression and overall survival in patients with small cell lung cancer and large cell neuroendocrine cancer. We also found that Klotho expression was associated with EMT-related molecules in lung squamous cell carcinoma. The present study aimed to analyze the function of the Klotho gene and to elucidate its relevance to the regulation of the EMT. For this purpose, GFP-Klotho plasmids were transfected into lung adenocarcinoma cells (A549) and cell lines with stable expression (A549/KL-1 and A549/KL-2) were established. A549/KL-1 cells expressed higher levels of Klotho protein by western blot analysis compared with A549/KL-2 cells. In western blotting of A549 and A549/KL-1 cells, the expression of the mesenchymal marker N-cadherin was found to be completely inhibited in A549/KL-1 cells suggesting that Klotho expression may regulate the EMT in cancer cells via the inhibition of N-cadherin. The results of the sensitivity tests demonstrated that A549/KL-1 cells were significantly more sensitive to pemetrexed compared with A549 cells (IC\textsubscript{50} A549/KL-1 vs. A549 cells, 0.1 \mu M vs. 0.7 \mu M). The results of the microarray analysis demonstrated that a very high level of lipocalin-2 (LCN2) expression was induced in the A549/KL-1 cells. Klotho overexpression completely suppressed the expression of mesenchymal markers, such as N-cadherin and Snail1 (Snail).

The results of the present study suggested that there may be a new mechanism of action for the antitumor effects of pemetrexed, namely, LCN2-mediated modulation of N-cadherin expression. Klotho expression during cancer treatment has great potential as a predictor for efficacy of pemetrexed and as a factor in the selection of personalized medicine for postoperative adjuvant chemotherapy.

Introduction

Lung cancer is the leading cause of cancer-related death in the world (1,2). The majority of these cancers are non-small cell lung cancers, of which adenocarcinoma is the most common histological subtype (1). However, treatment outcomes have been less than satisfactory. To improve the postoperative outcome of lung cancer, effective chemotherapy regimens need to be established for each individual case and individualized treatment practices need to be implemented. In recent years, new molecularly targeted drugs and immune checkpoint inhibitors have been developed and treatment strategies, especially for non-small cell lung cancer (NSCLC), have been improved using personalized medicine (2-4).

Epithelial-mesenchymal transition (EMT) is considered to serve an important role in the metastatic/invasive ability of cancer cells (5,6). As the EMT progresses, epithelial cells lose their cell-cell adhesion property and cell polarity and assume the phenotype of highly motile mesenchymal cells (5). E-cadherin is a typical adhesion molecule that forms the adhesion junctions between epithelial cells, and its expression is suppressed as EMT traits are acquired (6,7). In addition, increases in the expressions of the mesenchymal markers N-cadherin (a mesenchymal cadherin), vimentin (a cytoskeletal protein) and fibronectin (an extracellular matrix molecule) are observed during the EMT (8). In particular, among the cell adhesion molecules, the expression of the epithelial cadherin E-cadherin decreases during EMT, whereas the expression of the mesenchymal cadherin N-cadherin increases; this phenomenon is known as the cadherin switch and is thought to serve an important role in cancer cell invasion and progression (9,10). Since apoptosis resistance and stem cell properties also become manifest at the same time, EMT is considered to be a factor that generates diversity among cancer cells (11-14).
In addition, EMT causes cancer cells to become resistant to natural killer cells (15), and the induction of the EMT in cancer cells through the administration of anticancer drugs can lead to drug resistance (16-18). Thus, elucidating cancer diversity, resistance acquisition, and the related EMT mechanism could lead to new cancer treatment methods.

The Klotho gene was originally discovered during the creation of a hypertensive mouse model (19). Mice with genetic mutations in the Klotho gene exhibit a human-like aging-related syndrome and develop multiple disorders, including hypogonadism, ectopic calcification, osteoporosis, skin atrophy and pulmonary emphysema (20). Transgenic mice overexpressing Klotho have an extended life span that is 30% longer in males and 20% longer in females compared with wild-type controls (21). The Klotho gene is associated not only with antiaging, but also with cancer growth and invasion, including in breast, pancreatic, ovarian, lung, colorectal cancer and melanoma (22). Klotho functions as a tumor suppressor by inhibiting insulin/insulin growth factor 1, p53/p21 and Wnt signaling (23,24). The silencing of Klotho gene expression is mainly mediated through promoter hypermethylation and histone deacetylation in cancer (25). The Klotho gene reportedly acts as a tumor suppressor gene in breast cancer and suppresses proliferation and induces apoptosis in lung adenocarcinoma cells (26,27).

We hypothesized that the Klotho gene is also involved in the metastatic/invasive ability of lung cancer. We previously reported an association between Klotho expression and overall survival in patients with small cell lung cancer (SCLC) and large cell neuroendocrine cancer (LCNEC) (28,29). Klotho expression has also been shown to be an important postoperative prognostic factor in patients with SCLC and LCNEC (28,29). We also found that Klotho expression was associated with EMT-related molecules in lung squamous cell carcinoma (30).

The present study focused on the association between the Klotho gene and cancer metastasis in lung adenocarcinoma. In particular, the present study aimed to analyze the function of the Klotho gene to elucidate its relevance to the regulation of the EMT which is part of the metastasis process. The possibility of a new cancer treatment based on the Klotho gene using drugs and a growth-inhibition assay was also assessed. In particular, the present study focused on the use of pemetrexed, an antifolate drug (31). Studies have suggested that blocking the EMT pathway could eliminate resistance to antifolate chloride therapy in lung cancer (32). The findings of the present study that investigate the link between pemetrexed, EMT and the Klotho gene could be a stepping stone to new lung cancer therapies.

Materials and methods

Cell culture and transfection. A549 a human lung adenocarcinoma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. D6429; Millipore Sigma) supplemented with 0.1% sodium bicarbonate, L-glutamine, sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS; cat. no. F2442; Millipore Sigma), and penicillin (100 U/ml) in a humidified atmosphere of 5% CO₂ at 37°C. GFP-Klotho plasmids were transfected into A549 cells using the Lipofectamine 3000™ transfection reagent according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific Inc.). Cells were transfected with 2 µg of plasmid DNA using the transfection reagent, and were incubated for 2 h in a humidified atmosphere of 5% CO₂ at 37°C. The cells were washed and medium including FBS added (30,33). The GFP-Klotho plasmid or a GFP vector was transfected into the A549 cells, and 24 h later the cells were pelleted (almost 1 mg) by centrifugation at 1,500 x g for 10 min and resuspended in PBS to a final density of ~2.9x10⁶ cells/ml and the suspension was then filtered through a nylon membrane to remove cell aggregates (30). The GFP-positive cells were sorted using flow cytometry and a FACSCount II (BD Biosciences) with the activation set at 488 nm and fluorescence emission monitoring at 508 nm (GFP). The data acquisition and analysis were performed using FlowJo™ v.10.7 software (TreeStar Inc.). At least 10,000 events were collected for each analysis. Dead cells and debris were eliminated using the forward-scatter and side-scatter parameters, and the remaining cells were sorted into GFP-positive and GFP-negative populations.

Isolation of clones expressing Klotho. Following GFP-Klotho transfection, the GFP-positive cells were cultured with 0.1% sodium bicarbonate, L-glutamine, sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) in a humidified atmosphere of 5% CO₂ at 37°C for 2-3 weeks and the colonies were harvested. A single clone was obtained using a limiting dilution method (34). Several colonies were checked for Klotho expression using western blotting and two stably overexpressing Klotho cell lines: A549/KL-1 and A549/KL-2 were established.

Western blotting. A549, A549/KL-1, and A549/KL-2 cells were harvested by centrifugation at 1,500 x g for 10 min and washed twice with ice-cold PBS; the cell pellets were then incubated in a lysis buffer (50 mM Tris-HCl, pH 7.6, 120 mM NaCl, 1% Triton X-100, 0.2% sodium-deoxycholate and a protease inhibitor). After cooling the lysates on ice for 10 min, they were centrifuged at 14,000 x g for 30 min. For protein determination, a bicinchoninic Acid (BCA) kit was used (Sigma-Aldrich; Merck KGaA). Each sample of 10 µg of total protein was separated using 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated for 1 h at room temperature with the following antibodies: rat anti-human Klotho monoclonal antibody (1:500; clone KM2076; cat. no. KO603; Transgenic Inc.), rabbit anti-human E-cadherin monoclonal antibody (1:1,000; clone 24E10; cat. no. 3195), rabbit anti-human N-cadherin monoclonal antibody (1:1,000; clone D4R1H; cat. no. 13116), rabbit anti-human vimentin monoclonal antibody (1:1,000; clone D21H3; cat. no. 5741), or rabbit anti-human Snail monoclonal antibody (1:500; clone C15D3; cat. no. 3879) (all from Cell Signaling Technology, Inc.) or anti-TS antibody (clone 8F1; cat. no. 35-5800; Zymed; Thermo Fisher Scientific Inc.) (33,35). After rinsing with PBS containing 0.1% (v/v) Triton X-100, the membranes were incubated with goat anti-rat immunoglobulin-G (IgG)-conjugated horseradish peroxidase (HRP) (1:10,000 dilution; cat. no. 65-9520, Thermo Fisher Scientific Inc.) for the anti-Klotho antibody or...
with goat anti-rabbit IgG conjugated HRP (1:2,000 dilution; cat. no. 31460; Thermo Fisher Scientific Inc.) for the other antibodies for 1 h at room temperature. Blots were probed with β-actin (1:1,000 dilution; cat. no. 4967; Cell Signaling Technology, Inc.) as a loading control. The membranes were washed and developed with western blotting enhanced chemiluminescence detection reagents (Bio-Rad Laboratories Inc.) (36). The western blotting results were confirmed in three independent experiments.

Drugs and growth-inhibition assay. Afatinib, ceritinib, gefitinib and osimertinib were purchased from Selleck Chemicals. Carboplatin, cisplatin, docetaxel and paclitaxel were purchased from Sigma-Aldrich; Merck KGaA. Pemetrexed was purchased from Toronto Research Chemicals (37,38). A549 and A549/KL-1 cells (2,000 cells/well) were seeded into 96-well plates, and the drugs were added in increasing concentrations (0, 0.01, 0.1, 1, 10 and 100 µM) at room temperature. The half maximal inhibitory concentration (IC50) value was defined as the concentration of an anticancer agent required to reduce growth by 50%. The corrected absorbance of each sample compared with that of the untreated control was calculated by MTT assay using the absorbance wavelength at 570 nm.

Determination of cell viability. Sensitivity tests (MTT assays) for various anticancer agents including afatinib, carboplatin, ceritinib, cisplatin, docetaxel, gefitinib, osimertinib, paclitaxel and pemetrexed using A549 cells and A549/KL-1 cells were performed (39). DMSO was used to dissolve the purple formazan and measured by the wavelength at 570 nm.

cDNA microarray analysis. Gene expression in lung cancer cells, A549 cells and A549/KL-1 cells, were analyzed using a cDNA microarray. mRNA was extracted and purified using the mammalian total RNA Miniprep kit (cat. no. RTN10; Sigama-Aldrich Inc.). A commercially available cDNA microarray, the Human Genome U133 plus 2.0 array (cat. no. 900470, Thermo Fisher Scientific Inc.), on which 47,000 gene probes had been arrayed was used. Gene expression data was analyzed using Transcriptome Analysis Console (TAC) software v.3.1 (Applied Biosystems). Significantly modulated genes were defined as log2 fold change (FC)>2 and adjusted P-value<0.05.

Statistical analysis. Results of the experiments were presented as the mean ± SD or the mean of independent triplicate experiments and were analyzed using the Student's unpaired t-test. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft Corporation). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Klotho in A549 and stably overexpressing Klotho cell lines. To elucidate the mechanism of action of the Klotho gene, GFP-Klotho plasmids were transfected into the human lung adenocarcinoma cell line A549 and two stable cell lines were established: A549/KL-1 and A549/KL-2. In a western blot analysis these cell lines had high expression levels of Klotho protein, while the parental A549 cells did not express Klotho protein at all. The A549/KL-1 cells expressed higher levels of Klotho protein compared with A549/KL-2 cells (Fig. 1). Klotho expression is related to the sensitivity of cancer cells to pemetrexed. To evaluate the association between Klotho expression and the antitumor effect of various anticancer agents, sensitivity tests were performed to evaluate the growth inhibitory effects on both A549 cells and A549/KL-1 cells, which have high levels of Klotho expression (Table 1). The cell survival curves for A549 and A549/KL-1 exposed to
pemetrexed are shown in Fig. 3. Pemetrexed caused growth inhibition of A549/KL-1 at lower concentrations of pemetrexed compared with A549 (Fig. 3). The A549/KL-1 cells were considerably more sensitive to pemetrexed compared with the A549 cells, with IC$_{50}$ values of 0.1 µM in the A549/KL-1 cells and 0.7 µM in the A549 cells (P<0.001; Table I). However, no increased sensitivity of the A549 and A549/KL-1 cells was seen to the other anticancer agents tested, including the molecular-targeted drugs afatinib, ceritinib, gefitinib, and osimertinib and the cytotoxic drugs cisplatin, carboplatin, paclitaxel and docetaxel (Table I) (40). These results indicated that Klotho overexpression increased the sensitivity to pemetrexed, which is a multi-target antifolate that is active against various malignant tumors (31). There was a significant difference of IC$_{50}$ between A549 cells and A549/KL-1 cells in sensitivity to pemetrexed, but not the other anticancer agents (Table I). However, the mechanism through which Klotho expression affected the antitumor effect of pemetrexed was unclear, so next the expression of Klotho and EMT-related proteins after pemetrexed exposure were investigated. Notably, when A549 and A549/KL-1 cells were exposed to various doses of pemetrexed (0, 0.1 and 0.7 µM), Klotho expression increased in the A549/KL-1 cells in a dose-dependent manner, but Klotho expression was not observed in the A549 cells (Fig. 4). These findings suggest that Klotho expression is related to the sensitivity of cancer cells to pemetrexed.

Klotho expression strongly suppresses N-cadherin expression. N-cadherin was completely repressed in A549/KL-1 cells compared with the parental cell line, regardless of...
pemetrexed concentration, but no difference was observed in other skin-mesenchymal transition-related proteins, such as E-cadherin and vimentin (Fig. 5). In A549/KL-1 cells, compared to the parental cell line, Snail expression was slightly reduced by pemetrexed exposure (Fig. 5). Thymidylate synthase (TS) is a target of antifolate drug expression (41). Pemetrexed is a novel antifolate that inhibits several folate-dependent enzymes in addition to TS (31). However, TS expression was slightly increased by pemetrexed exposure, but no difference in TS was observed between the A549 and A549/KL-1 cells (Fig. 6). In A549/KL-1, N-cadherin expression was completely suppressed and N-cadherin was not expressed when the cells were exposed to pemetrexed (Figs. 2 and 5). Hence, Klotho expression strongly suppressed N-cadherin expression.

Klotho gene expression may play a significant role in the regulation of LCN2. To further assess whether Klotho transfection affects host gene expression, microarray analysis was performed to study host genes with differential expression in A549 and A549/KL-1 cells. The top 30 genes with high-fold change values were assessed and the results demonstrated that the expression of EMT-related genes (discoidin I-like domain-containing protein 3 (EDIL3), pentraxin-3 (PTX3), fibrillin-2 (FBN2), interleukin-6 (IL-6)) and genes related to pemetrexed (dihydropyrimidine dehydrogenase (DPYD), lipocalin-2 (LCN2)) (44) was upregulated by the introduction of the Klotho gene (Fig. 7). Downregulated genes were also evaluated, but there were no genes that appeared to be involved in EMT or in the suppression of cadherin (data not shown). These results suggested that Klotho gene expression may play a significant role in the regulation of LCN2.

Discussion

We previously reported that the overexpression of Klotho almost completely suppressed N-cadherin expression using a transient model (30), but the mechanism responsible for the regulation of the EMT by N-cadherin remained unknown. In the present study, the A549/KL-1 cell line with stable Klotho overexpression was established. It was reported that Klotho inhibited TGF-β1-induced EMT responses (45,46). Also, it was demonstrated that Klotho is capable of inhibiting the activation of the Wnt signaling pathway, which inhibits glycogen synthase kinase-3β (47,48). Based on the above findings, it was speculated that Klotho serves a critical role as a suppressor of N-cadherin expression by inhibiting Wnt and TGF-β1 signaling.

The results of the present study demonstrated that the expression of EMT-related genes (EDIL3, PTX3, FBN2 and IL-6) and genes related to pemetrexed (DPYD, lipocalin-2 (LCN2)) was upregulated by the introduction of the Klotho gene. In the present study, LCN2 was focused on among the upregulated genes, which has been reported to be associated with EMT and pemetrexed. LCN2 belongs to the lipocalin protein family and can exist as a 25-kDa monomer or a 46-kDa disulfide-linked homodimer (44). LCN2 reportedly suppresses cellular invasion and metastases and blocks EMT (49). Wang et al (50) demonstrated that LCN2 negatively modulates EMT and suppresses N-cadherin expression.
Figure 7. Table of the top 30 genes with high fold change values with microarray analysis. Data are presented as a fold change in relative gene expression between A549 and A549/KL-1 cell lines. KL, Klotho.

Figure 8. Schematic diagram of the mechanism of action of pemetrexed and the regulation of EMT dependent upon the expression of the Klotho gene. EMT, epithelial mesenchymal transition; Snail, Snail 1.
in vitro. Feng et al (51) found that LCN2 suppresses proliferation, metastasis/invasion and EMT by attenuating the promoter activity of the NF-xB-dependent activation of Snail. The present study also assessed downregulated genes, but there were no genes that seemed to be involved in EMT or suppression of cadherin.

We therefore hypothesized that the overexpression of Klotho completely suppressed the expression of the mesenchymal markers N-cadherin and Snail through the induction of LCN2, as shown in Fig. 8. Based on the findings of the present study, it seems that Klotho expression does not directly inhibit EMT, but that LCN2 upregulates Klotho by inhibiting EMT. How LCN2 suppresses and modulates N-cadherin and Snail remains unknown and this needs further investigation by future studies.

The induction of Klotho expression by pemetrexed treatment is a very interesting phenomenon. Hence, it was hypothesized in the present study that if Klotho expression is a determinant of pemetrexed sensitivity, pemetrexed may suppress cell proliferation by regulating N-cadherin and Snail through the induction of LCN2. It was reported that microRNA (miR)-761 overexpression reduced the expression of N-cadherin and vimentin, but increased the expression of E-cadherin suggesting inhibition of EMT (52). Tao et al (53) demonstrated that miR-451a serves a significant role in suppressing the drug resistance of lung cancer cells treated with doxorubicin by alleviating the effect on the EMT, as evidenced by marked reductions in the expressions of N-cadherin and vimentin. In addition, Ren et al (54) demonstrated that wild-type p53 suppresses migration, invasion, EMT and stemness in PC-3 cells, at least partially by modulating miR-145.

TS as one of the targets for antifolate drugs was added to the analysis of the present study. Pemetrexed upregulated the expression of TS and this finding was consistent with the results of previous studies (55-57). Although pemetrexed treatment increased the expression of TS in both the A549 and A549/KL-1 cells in the present study, no difference in TS expression was observed between the two cell types. Hence, pemetrexed, which is a folate metabolism antagonist in Klotho-overexpressing cells (44), may activate mechanisms other than those involving TS. The involvement of EMT regulation as a determinant of pemetrexed activity has been less clear, but the involvement of the EMT in resistance has been previously suggested (44). Wu et al (49) demonstrated that pemetrexed caused cell cycle arrest in the G1 phase and S phase of A549 cells, and they noted a high expression of LCN2 proteins in A549 cells after pemetrexed exposure. The aforementioned study concluded that LCN2 can be used as a new biomarker for predicting the responsiveness to pemetrexed (44). Such reports are not inconsistent with the schematic proposed by the present study (Fig. 8).

To the best of our knowledge, this is the first study that found that the Klotho gene was a suppressor of the EMT in lung adenocarcinoma. However, there were several limitations of the present study including that only the A549 cell line was used, how LCN2 suppresses EMT via Klotho overexpression was not clarified and genes downregulated by Klotho expression were not investigated. Hence, further studies are needed to draw more definitive conclusions about the association between Klotho expression and LCN2 and the effect of LCN2 on EMT in lung adenocarcinoma. In addition, how LCN2 suppresses and modulates N-cadherin and Snail also warrants future investigation.

The results of the present study reveal a novel mechanism of action on the antitumor effects of pemetrexed: Namely, the regulation of LCN2-mediated N-cadherin expression. In addition, confirming Klotho expression during cancer treatment may have great potential as a predictor of pemetrexed efficacy and as a component in the selection of personalized medicine for adjuvant chemotherapy in postoperative settings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JU conceived and designed the experiments. The experiments were performed by KT. KT, TI, TS, TM and MM provided substantial contributions to the analysis of the data and creation of the figures. JU and KT wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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