Increased 18F-FDG uptake and expression of Glut1 in the EMT transformed breast cancer cells induced by TGF-β

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As carcinomas progress, the tumors may lose epithelial morphology and acquire mesenchymal characteristics typically called epithelial-mesenchymal transition (EMT), which is commonly associated with increased cell migration, enables cells to dissociate from their original tissue and form metastasis in distant organs. In addition to molecular and morphologic changes, the EMT transformed cells also showed the change of sensitivity to chemotherapeutics. In order to detect the EMT transition in vivo clinically, we detected the change of metabolism of MCF-7 cells after being induced by TGF-β to form EMT condition by MTT and 18F-FDG uptake.

Key words EMT; TGF-β; breast cancer; metabolism; 18F-FDG

Epithelial-mesenchymal transition (EMT) refers to the transdifferentiation of epithelial cells to a more mesenchymal state, a series of phenotypic and molecular changes that occur in various steps of normal development and in cancer cells. Reductions of epithelial cell-cell adhesion via the transcriptional repression of cadherins in combination with the acquisition of mesenchymal properties are key determinants of EMT. When EMT occurs in cancer, the prognosis may be adversely affected [1]. As carcinomas progress, these tumors may lose epithelial morphology and acquire mesenchymal characteristics which contribute to metastatic potential.

EMT is characterized by several molecular changes that include the loss of epithelial markers such as E-cadherin, these intercellular junctions are down-regulated, leading to the loss of epithelial coherence [2–5], and the induction of mesenchymal markers such as vimentin, N-cadherin, fibronectin, and Snail [4]. In EMT process, epithelial cells detach from the extracellular matrix (ECM), acquire fibroblast-like properties, and show reduced intercellular adhesion and increased motility [6, 7]. The reversal of EMT in cancer cells can decrease the invasive ability.

In addition to several molecular and morphologic changes, the EMT transition cells also showed the variation of sensitivity to chemotherapeutics. Epithelial tumor cells have been shown to be significantly more sensitive to EGFR inhibitors than tumor cells which have undergone an EMT-like transition and acquired mesenchymal characteristics, including non-small cell lung, head and neck, bladder, colorectal, pancreas and breast carcinomas [8]. So the EMT condition of carcinoma is related to the therapy of patients, however, in previous researches, almost all assessments about EMT are based on the morphology change and the change of molecular changes such as E-cadherin, vimentin, N-cadherin, fibronectin, and Snail in tumor cell or in excisional tumor samples. Positron emission tomography (PET) is a multi-purpose non-invasive imaging technique with a wide range of applications both in vivo and in vitro [9]. In clinical oncology PET has been used for diagnosis, staging, and restaging after treatment or recurrence of different malignancies, including breast cancer [10–12]. In this study, we want to detect the metabolic change of MCF-7 cells after the EMT transition induced by TGF-β by PET and MTT methods, and supply the evidences for evaluating the EMT condition by clinically imageology more easily.

Materials and methods

Cell culture. MCF-7 cells were cultured in RPMI-1640 (HyClone) with 10% FBS (Gibico), penicillin, and streptomycin. The cells were incubated at 37° C in humidified air with 5% CO₂. The medium was replaced thrice weekly, and cells were
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maintained by serial passage after trypsinization with 0.1% trypsin. For the experiment group, MCF-7 cells were cultured in the RPMI-1640 with 10% FBS, penicillin, and streptomycin, in addition with 80 μmol/L TGF-β1 (R&D systems) for 72 h.

Western blot assays. Cells were cultured in growth medium on 35-mm tissue culture plates to approximately 80% confluence. After briefly rinsing with PBS, the cells were lysed in ice cold buffer containing 1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 200 μg/ml PMSF and 50 μg/ml aprotinin according to the manufacturer’s instructions. The cell lysates were subjected to brief sonication and heated for 5 minutes at 100°C. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes, which were blocked with 5% nonfat dry milk in TBS for 2 hours and then incubated with the primary antibodies. Afterwards, with horseradish peroxidase-conjugated rabbit antimouse IgG. As control for equivalent protein loading, filters were simultaneously incubated with a mouse MAb directed against β-actin (Chemicon). The protein-antibody complexes were visualized by ECL chemoluminescence.

Immunofluorescence. MCF-7 cells were seeded onto round gelatin coated glass coverslips placed in 24-well plates and stimulated with recombinant TGF-β1 for an addition 72 h. cells were fixed with ice cold methanol for 10 min, permeabilized with 0.2% Triton for 3 min and blocked in 10% goat serum for 1h at room temperature. E-cadherin, vimentin and Glut-1 (Maxim) was probed with first antibody for 1h at room temperature followed by detection with a rhodamine-conjugated goat anti-mouse secondary antibody for 1h at room temperature. Hoechst dye (1μg/ml) was subsequently used to stain nuclei.

Cell viability assay. The MTT assay was used to determine viable cell metabolism. Briefly, MCF-7 cells were plated in 96-well microtiter plates (1000 cells/well) and the cells were allowed to incubate for 24, 48, and 72 h. 20 μl MTT (5 mg/mL in PBS; Sigma) was added, and the cells were incubated for 2 h. After removal of the medium, 100 μL buffered DMSO was added to each well, and the plates were shaken. Absorbance at 570 nm (proportional to viable cell number) was then read with a multiplate reader (Bio-Rad Laboratories).

Transwell invasion assays. For invasion assays, 6.5 mm Costar Transwell chambers with 8-µm pores were used [13]. The Transwell membranes were precoated with 20% FBS on the underside only for 2 h at 37°C. 5×10^4 cells in 200 µl of complete medium were seeded into the upper chamber. 0.6ml of medium was added to the lower chamber, and the plate was incubated at 37°C in a 5% CO₂ incubator for 24 hours added to the lower compartment. After 12 h of incubation, the migration of the cells was determined by counting the number of cells that migrated through the pores to the lower side of the filter using a microscope at 100×magnification. The experiment was performed for three times.

$^{18}$F-FDG uptake. Approximately 5×10^5 MCF-7 cells were seeded into 6-well plates, and allowed to attach overnight. One hour before the appropriate testing point, the cells were washed with PBS and given low-glucose DMEM medium. The MCF-7 cells were incubated for one hour at 37°C with 0.5 ml medium per well containing 3MBq $^{18}$F-FDG, after this uptake phase, the medium containing the $^{18}$F-FDG was removed and the cells were washed twice with ice-cold PBS (1 ml/well). Finally cells with 20 μl washing medium were transferred to 5 ml tubes and $^{18}$F-FDG uptake was evaluated in a calibrated well γ-counter. A 20 μl sample of the incubation medium was measured as reference, and 20 μl from the last wash medium was measured as background control [14, 15].

Statistical analysis. For each condition, the data were presented as mean ± standard deviation (SD) and analyzed using the SPSS13.0. Analysis of variance was conducted, followed by independent-samples t test. P value less than 0.05 was considered statistically significant.

Results

Morphology change after EMT transition. In this study, TGF-β1 was used to induced EMT of MCF-7 cells, with 72 hours of TGF-β1 treatment, MCF-7 cells undergo a dramatic morphological change, from compact, cobblestone-like epithelial structures to fibroblastoid spindle-shaped cells, with prominent longitudinal stress-fibers, consistent with amyofibroblast-like phenotype, together with significant disintegration of cell-cell adhesions (Fig.1).

Down regulation of E-cadherin, and upregulation of vimentin and Glut-1 proteins. EMT process involves losing of epithelial

Fig.1 The morphological change of MCF-7 cells induced by 80 μmol/L TGF-β1 for 72h
phenotype and obtaining some specific characteristics of mesenchymal cells. As observed, this morphological transition is accompanied by E-cadherin down-regulation with reduced membrane localization in the MCF-7 cells treated with TGF-β1. Moreover, the expression of vimentin was up-regulated in MCF-7 cells treated with TGF-β1, whereas its expression was markedly short in the MCF-7 cells (Fig. 2). These hallmark shifts at the morphological and molecular levels indicate a successful EMT program in MCF-7 cells. Additionally, we also showed that downregulation of E-cadherin and upregulation of vimentin and Glut-1 with Western blot consistent with the results of immunofluorescence (Fig. 3).

*Increased migratory ability after TGF-β1 treatment.* EMT is thought to promote cancer cell migration and invasion, in order to examine whether TGF-β1 treatment could affect cell motility, an in vitro migration assay was performed, 10% FBS was added to the bottom chamber as a chemoattractant. The numbers of MCF-7 cells that migrated through the filter was increased 1.9 ± 0.1-fold in TGF-β1 treated group cells (P < 0.01).

*Change of cell metabolism and 18F-FDG uptake alteration.* By exposing 20, 80 and 320 μmol/L TGF-β1 for 24 h, 48, and 72 hours, in order to calculate the standard metabolic rate of per cell, the ratio value of MTT metabolism value to the cell number was obtained and the ratio value of control group was regarded as 1; the standard metabolic rate increased after the EMT transition of MCF-7 cells cultured with TGF-β1, with significant difference compared to the control groups (P < 0.01) (Fig. 4).

18F-FDG uptake values per cell and per well were obtained and are reported in Figure 5 and given as a percentage of the values at time 0. As expected, the 80μmol/L TGF-β1 treated MCF-7 cells showed the gradually elevated 18F-FDG uptake per cell with the prolonged effect of TGF-β1 compared to the untreated group (P < 0.01) (Fig. 5).

**Discussion**

Epithelial-mesenchymal transition (EMT) is essential developmental processes by which cells of epithelial origin lose cells lose cell-cell adhesion and polarity, and acquire a mesenchymal phenotype and obtaining some specific characteristics of mesenchymal cells. As observed, this morphological transition is accompanied by E-cadherin down-regulation with reduced membrane localization in the MCF-7 cells treated with TGF-β1. Moreover, the expression of vimentin was up-regulated in MCF-7 cells treated with TGF-β1, whereas its expression was markedly short in the MCF-7 cells (Fig. 2). These hallmark shifts at the morphological and molecular levels indicate a successful EMT program in MCF-7 cells. Additionally, we also showed that downregulation of E-cadherin and upregulation of vimentin and Glut-1 with Western blot consistent with the results of immunofluorescence (Fig. 3).

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**Discussion**

Epithelial-mesenchymal transition (EMT) is essential developmental processes by which cells of epithelial origin lose cells lose cell-cell adhesion and polarity, and acquire a mes-
Enhancement of cell migratory behavior [16, 17]. The most compelling evidence for the involvement of EMT in oncogenesis is the ability of multiple EMT regulators to enhance tumor formation and/or metastasis. EMT is commonly associated with increased cell migration, which enables cells to dissociate from their original tissue and form metastasis in distant organs. The occurrence of EMT during tumor progression allows tumor cells to acquire the capacity to infiltrate surrounding tissue and to ultimately metastasize to distant sites [18–20], EMT have been implicated in tumor recurrence [21], and are often associated with a poor prognosis in women with breast cancer [22–24].

Furthermore, EMT is characterized by loss of intercellular adhesion; down-regulation of epithelial makers; up-regulation of mesenchymal markers, such as vimentin and smooth muscle actin (SMA); acquisition of fibroblast-like morphology with cytoskeleton reorganization; and increase in motility, invasiveness, and metastatic capabilities. Tumor cells undergoing EMT have been shown to undergo 'cadherin switching', downregulating E-cadherin and compensating with alternate cadherin
proteins such as N-cadherin [25–27] and the accumulation of h-catenin have also been associated with EMT [6, 28].

Epithelial-to-mesenchymal transitions have been induced in vitro by transient or unregulated activation of receptor tyrosine kinase signaling pathways, oncogene signaling and disruption of homotypic cell adhesion. TGF-β1 is a potent inducer of EMT [29]. It not only contributes to EMT during embryonic development but also induces EMT during tumor progression in vivo [30]. In this study, TGF-β1 was used to induced EMT of MCF-7 cells. With 72 hours of TGF-β1 treatment, MCF-7 cells undergo a dramatic morphological change, from compact, cobblestone-like epithelial structures to fibroblastoid spindle-shaped cells, together with significant disintegration of cell-cell adhesions. Except for the morphological change, as observed, this morphological transition is accompanied by E-cadherin down-regulation with reduced membrane localization in the MCF-7 cells treated with TGF-β1. Moreover, the expression of vimentin was up-regulated in MCF-7 cells treated with TGF-β1, whereas its expression was markedly short in the MCF-7 cells. These hallmark shifts at the morphological and molecular levels indicate a successful EMT program in MCF-7 cells. Additionally, we also showed that downregulation of E-cadherin and upregulation of vimentin by Western blot consistent with the results of immunohistochemistry. Many mechanisms are involves the TGF-β1 inducing EMT, transcriptional repressors of the E-cadherin gene, such as members of the Snail family of zinc finger proteins Snail, Slug), two-handed zinc finger/homeodomain proteins ZEB1, ZEB2, Twist and LEF-1 are involved in the EMT response to TGF-β [31]. TGF-β cooperates with numerous kinases such as RAS, MAPK, p38 to promote EMT [32].

EMT is not only involved in morphologic change, but also lead carcinoma cell insensitivity to chemotherapeutics. The detection of EMT signatures is not only important for diagnosis but can also be exploited to enhance classical chemotherapy treatments. Activation of the epidermal growth factor receptor (EGFR) has been implicated in the neoplastic transformation of solid tumors and overexpression of EGFR has been shown to correlate with poor survival. Notably, epithelial tumor cells have been shown to be significantly more sensitive to EGFR inhibitors than tumor cells which have undergone an EMT-like transition and acquired mesenchymal characteristics [33, 34]. These observations were later extended to other tumor types and EGFR antagonists, including pancreatic, colorectal [35], head and neck [36], bladder [37] and breast [38] suggesting that EMT status may be a broadly applicable indicator of sensitivity to EGFR inhibitors. Gain of N-cadherin expression is a typical example of the EMT signature and it has been linked with drug resistance [39]. It has been shown that most basal-like breast cancer is in EMT condition, and the tumors show a special proclivity for distant metastasis to characteristic tissues [40], basal-like breast cancers is usually related to positive of HER2 and negative of ER and PR and show unfavorable prognosis and/or resistance to chemotherapy [41, 42].

So analysis of EMT condition plus histochemical analyses in breast cancer cells provides a good model to evaluate prognosis. Positron emission tomography (PET) is a multi-purpose non-invasive imaging technique with a wide range of applications both in vivo and in vitro [11, 43–45]. In clinical oncology PET has been used for diagnosis, staging, and restaging after treatment or recurrence of different malignancies, including breast cancer. In order to explore if EMT transformation of MCF-7 cells could impact on the cellular uptake of 18F-FDG, and detected the relationship between EMT and the metabolic of carcinoma cell, we utilized TGF-β to treated MCF-7 cells and evaluated the uptake of 18F-FDG, 1.68, 2.15, and 2.3 fold of the standard metabolic rate was increased in 40, 80 and 160 μmol/L TGF-β respectively treated group for 72 h compared to control group. 1.35, 1.59 and 1.86 fold of the uptake of 18F-FDG was increased in 80 μmol/L TGF-β 72 h late, with the increase of TGF-β concentration, the uptake of 18F-FDG increase accordingly. This feature could be used for screening and selecting 18F-FDG PET biomarkers for early assessment of treatment response.

To explore the mechanism that may involve in the increase uptake of 18F-FDG when cancer cells are experience the transition of EMT, we further detect the expression of Glut-1 by western blot and immunofluorescence, a transporter located on the membrane of tumor cells. When the MCF-7 cell treated with TGF-β, the expression of Glut-1 increase, this indicates that the overexpression of Glut-1 increase in vivo transition MCF-7 cells.

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