EVEROLIMUS-INDUCED EPITHELIAL TO MESENCHIMAL TRANSITION (EMT) IN BRONCHIAL/PULMONARY CELLS: WHEN THE DOSAGE DOES MATTER IN TRANSPLANTATION.

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OBJECTIVES

• Although its clinical utility, as other antineoplastic/immunosuppressive drugs, Everolimus (EVE) may induce the development of systemic side effects including severe fibro-interstitial pneumonitis (e.g., bronchiolitis obliterans with organizing pneumonia and focal pulmonary fibrosis) [1,2]. The exact molecular/biological mechanism associated to these pro-fibrotic effects is unknown, but epithelial to mesenchymal transition (EMT) may have a central role.

• EMT is a process by which cells undergo a morphological switch from the epithelial polarized phenotype to the mesenchymal fibroblastoid phenotype [3].

METHODS

• ***Patients:*** 26 renal transplant recipients were included in the study. Patients were in maintenance treatment with Everolimus (EVE, n:13) or Tacrolimus (Advagraf-ADV, n: 13) plus methylprednisolone (4 mg/day) and mycophenolic acid (720 mg/day).

• ***Pulmonary fibrosis index score (PFIS):*** For all patients included in the study, PFIS was defined as the sum of three singular scores (lung, functional and alveolar score). **Lung score** was defined according to the evaluation of CT imagines. **Functional score** was assessed by function tests. **Alveolar score** was measured according to the hemogasanalysis.

• ***Cell Culture:*** Human NSCLC cell line **A549 (pulmonary adenocarcinoma cell line)**, **normal bronchial epithelial cell line called NuLi-1**, and a **bronchial epithelial cell line homozygous for the delta F508 cystic fibrosis-causing mutation (Cufi-1)** were grown according to our protocol. Then, cells were grown to sub-confluence, starved in serum-free medium for 24 hours and then cultured in serum-free medium with or without 5, 10, and 100nM EVE or 5, 500nM and 5microM TAC. As positive control, we used the effects induced by treatment with TGFBeta-1, a well-known EMT-inducer.

• ***Gene expression analysis*** **:** Real-time PCR for **alpha-smooth muscle actin (α-SMA), Fibronectin (FN) and Vimentin (VIM) and normalized to GAPDH.** was performed on an ABI-Prism 7500 using Power SYBR Green Master Mix 2X (Applied Biosystems).

• ***Immunofluorescence:*** After 48h of treatment with EVE or TAC, cells were incubated with primary antibodies for alpha-SMA (Sigma), VIM (Santa Cruz) and FN (Santa Cruz).

RESULTS

FIGURE 1

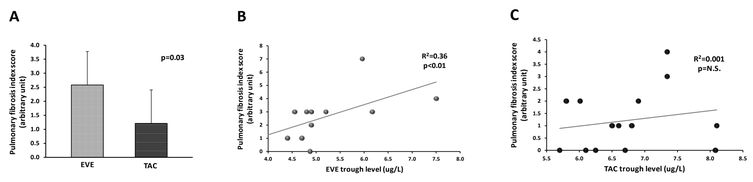


FIGURE 2

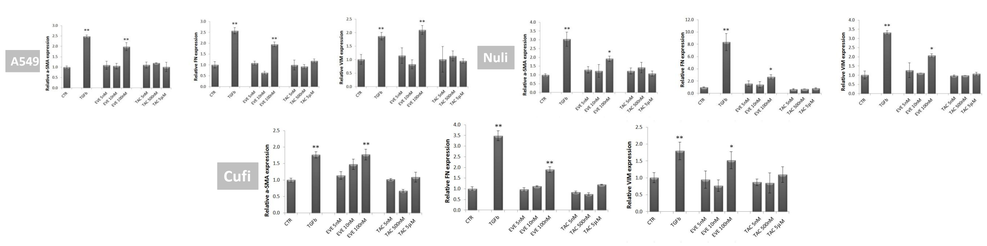
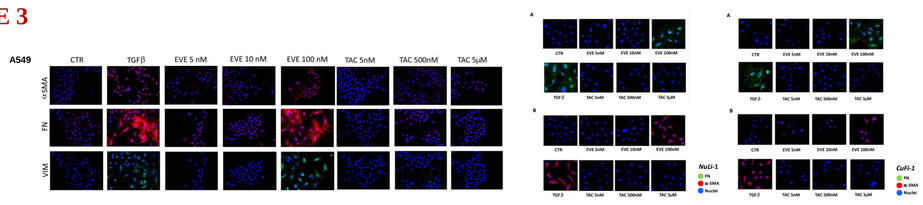


FIGURE 3



Pulmonary fibrosis score (PFIS) and correlation with EVE trough blood levels. As showed in **FIGURE 1** PFIS levels resulted significantly higher in EVE-treated patients compared to TAC-treated patients. Additionally, in EVE group PFIS was significantly correlated with the trough blood levels. These data suggested that the magnitude of chronic exposure to the immunosuppressive drug may have a central role in the pathogenesis of fibrotic-related pulmonary complications.

Gene expression analysis confirmed the in vitro ability of high dose EVE to induce EMT in bronchial cell lines. Interestingly, only high concentration of EVE (100 nM), similarly to TGF-B (20 ng/ml), were able to enhance the expression level of α-SMA, VIM and FN in these cell lines. Moreover, 10nM EVE did not induce any change in αSMA, VIM and FN mRNA levels. These effects were absent in the same cellular model treated with different dosages of TAC (**FIGURE 2**).

Up-regulation of the protein level of EMT markers after treatment with high doses of EVE.

Concordantly to RT-PCR experiments, immunofluorescence analysis showed that high concentration of EVE (100nM) increased protein expression of α-SMA, VIM and FN in A549, NULI and CUFI. Additionally, cells treated with 10 nM EVE did not show any change in the protein expression of the above mentioned mesenchymal markers. Also at protein level, TAC did not induce up-regulation of any analyzed fibrosis marker (**FIGURE 3**).

CONCLUSIONS

In our report, we found minimal and moderate fibrotic lesions and functional alterations in a group of asymptomatic EVE-treated patients undergoing screening morphological and functional lung tests. On the contrary, in the TAC-treated group, the lung involvement was lesser evident.

Our results then confirmed our in vitro finding reporting that only very high doses of EVE were able to induce up-regulation of alpha-SMA, Fibronectin and Vimentin at gene-expression and protein level in A549, NULI and CUFI. Additionally these biological effects were dose-correlated in both in vivo and in vitro model demonstrating the importance to moderate drug dosage.

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