

## **In vitro study of potential pro-fibrotic effect of Everolimus in different human airway cell lines. Searching for new biomarkers to optimize MTOR-inhibitor immunosuppressive treatment of cystic fibrosis patients undergoing lung transplantation**

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**Background.** MTOR-inhibitors (mTOR-I), Everolimus (EVE) and Sirolimus, immunosuppressants broadly used in transplantation, may determine severe adverse events including pulmonary fibrosis.

**Hypothesis and objectives.** The pathogenic mechanism of mTOR-I-associated pulmonary toxicity is still unclear, but epithelial to mesenchymal transition (EMT) of bronchial/pulmonary cells may have a role.

**Methods.** Human type II pneumocyte-derived A549, normal (Nuli-1) and homozygous for the delta F508 mutation causing cystic fibrosis (Cufi-1) bronchial epithelial cell lines were treated with EVE or Tacrolimus at different concentration. RT-PCR and immunofluorescence were used to evaluate mRNA and protein levels of EMT markers ( $\alpha$ -SMA, Vimentin, Fibronectin). Subsequently transcriptomic profile has been performed on Nuli-1, Cufi-1, primary bronchial epithelial cells (BE 63/3) and homozygous for the delta F508 mutation causing cystic fibrosis (BE 91/3) treated with EVE 5 nM and 200 nM. Finally, in 13 EVE- and 13-Tacrolimus-treated patients we compared the rate of lung fibrosis, estimated by an arbitrary pulmonary fibrosis index score (PFIS).

**Results.** Biomolecular experiments demonstrated that high dose of EVE (100 nM) up-regulated EMT markers in all cell lines at both gene- and protein level with a significant AKT-phosphorylation. Transcriptomic analysis revealed that EVE (5 nM) caused up-regulation of 29 genes in Nuli-1, 10 genes in Cufi-1, 19 genes in BE 63/3 and 86 genes in BE 91/3.

EVE (200 nM) caused up-regulation of 23 genes in Nuli-1, 13 genes in Cufi-1, 47 genes in BE 63/3

and 42 genes in BE 91/3. Interestingly this analysis confirmed the first part of the study revealing the upregulation of several EMT related genes (e.g., MMPs, COL12A1) only in cells exposed to high dose of EVE.

In the in vivo part of the study, we found that the PFISs were significantly higher in EVE-group compared to Tacrolimus-group ( $p=0.03$ ) and correlated with trough levels ( $R^2=0.35$ ).

**Spin-offs for research and clinical purposes.** Our data revealed, for the first time, a dose-dependent EVE-induced EMT in airway cells. Additionally, they suggest that clinicians should employ, whether possible, low dosages of mTOR-I evaluating periodically pulmonary function. Our genetic profile, in future, whether validated in patients, could be useful for clinicians to personalize mTOR-I treatment in solid organ transplant recipients minimizing lung toxicities.

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