

Human bronchial epithelial cells as a good control for evaluation potential therapeutic Notch signaling in non-small cell lung cancer

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ABSTRACT

Purpose: Notch signaling is often deregulated in non-small cell lung cancer (NSCLC), but little is known about the initial endogenous mRNA status of Notch ligands and receptors. Therefore, the aim of this study was to evaluate expression level of NOTCH1 receptor and Notch ligands, such as delta like ligands (DLL1, DLL3, DLL4), and jagged ligands (JAG1, JAG2), as well as target gene-hes family bHLH transcription factor 1 (HES1) in diverse NSCLC cell lines.

Materials and Methods: We have investigated the mRNA expression of chosen genes by using quantitative real time method (RT-PCR). We compared the results from NSCLC cells with results obtained in non-cancerous human bronchial epithelial cells (HBEPc). We also measured NOTCH1 expression in A549 cells, before and after treatment with γ -secretase inhibitor (GSI).

Results: The expression level of NOTCH1, HES1, JAG1 and JAG2 was downregulated when compared to HBEPc. The expression of Notch ligand DLL1 was lower in all cancer cell lines, but mRNA level of DLL3 was elevated in H1299 and A549 cells when related to HBEPc. The mRNA level of DLL4 was higher in H520 and in A549 cell lines. Moreover, the mRNA level of NOTCH1 dropped down after GSI treatment, in addition A549 cells proliferated slower after drug implementation.

Conclusions: We conclude that non-cancerous HBEPc cells could serve as a good control for Notch mRNAs expression analysis in NSCLC. Moreover, GSI-treated cells could inhibit proliferation through suppressing NOTCH1 in A549 cells.

Keywords: Notch, NSCLC, HBEPc

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INTRODUCTION

Lung cancer is one of the most mortal cancers all over the world. Regardless of therapeutic innovation, the low overall survival ratio did not change during past years [1,2]. Histologically, lung cancer consists of diverse tumor cells, including non-small cell lung cancer (NSCLC). Moreover, it is thought that NSCLC tumor subtypes derive from different sections of the airways [3]. Therefore, three major forms of NSCLC can be differentiated: adenocarcinoma, squamous cell (epidermoid) carcinoma and large cell (undifferentiated) carcinoma. Additionally, adeno-squamous carcinoma and sarcomatoid carcinoma subtypes of NSCLC exist, but those are much less common. NSCLC is characterized by an extensive local invasion, prompt clinical progression and poor prognosis. From a clinical perception, it is extremely important to understand the molecular pathogenesis of lung cancer and find novel biomarkers of prognosis. Notch signaling is engaged in lung cancer pathogenesis and consists of four Notch receptors (NOTCH1, 2, 3, 4) and five Notch ligands (DLL1, 3, 4 and JAG1, 2). Thus, it is extensively studied pathway in lung cancers; the overall status of Notch receptors and ligands in NSCLC still raises many questions [4,5]. According to the literature, there are wide-ranging results indicating very different mRNA expression status of Notch signaling genes in lung cancer. Moreover, it has been thought that Notch signaling can have oncogenic or tumor suppressive effect [6].

Therefore, in this study, we show that Notch signaling is active in NSCLC cells and inhibition of NOTCH1 can decrease proliferation of A549 cells. Moreover, we have evaluated the way to measure the actual mRNA expression level in NSCLC cells while using non-cancerous human bronchial epithelial cells as a control. In our research we also wanted to point out that a proper determination of Notch genes expression in NSCLC cells depends on many factors, including: specific control, tumor cell type, as well as growth factors in the microenvironment.

MATERIALS AND METHODS

Cell lines

The HBEpC cell line used in this study was purchased from European Collection of Authenticated Cell Cultures (ECACC) operated by Public Health England and was grown in bronchial epithelial cell serum free growth medium (BEGMTM, Lonza), supplemented with retinoid acid in 5%CO₂ at 37°C.

The human NSCLC cell lines were obtained from the American Type Culture

Collection (ATCC) and were grown in RPMI-1640 (H520, H1299), in EMEM (Calu-3) and in Ham's F12K medium (A549), supplemented with 10% fetal bovine serum (Sigma) in 5%CO₂ at 37°C. Media for NSCLC were purchased in ATCC. A549 cells were treated with γ -secretase inhibitor - RO4929097 (Selleckchem) with final concentration of 20 μ M. The experiment was performed in six well plates. The treatment was conducted for 24, 48 and 72 hours. Measurement of NOTCH1 expression was performed before and at 24, 48 and 72 hours of treatment. Microscopic evaluation of the cells proliferation status was performed with kinetic proliferation assay using label free cell counting by Cytation3 Multi-Mode Reader (Biotek).

RNA extraction and quality control

Total RNA was isolated from all NSCLC cell lines and HBEpC by using the RNeasy Kit following the manufacturer's protocol (Qiagen, USA). The 100 μ l RNA extracts were stored at -80°C prior to further processing. Quantity and quality of RNA assessment were performed using a UV/VIS spectrophotometer Nano Drop 2000c (Thermo Fisher Scientific, Inc., USA). The level of integrity required for quantitation was determined for the extracted total RNA using the Agilent RNA 6000 Nano kit on a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufactures' recommendations. Finally, a total of 400 ng of the RNA was reverse transcribed into cDNA in a reaction with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., USA) according to the manufacturer's protocol.

Quantitative polymerase chain reaction (qPCR)

The mRNA expression level of chosen genes was evaluated in NSCLC and HBEpC cell lines using quantitative real time PCR (RT-PCR). For real time reaction the TaqMan commercially available Gene Expression assays (Applied BiosystemsTM, USA) were used (Table 1).

The expression of the above-mentioned genes [by the change-in-cycling-threshold Δ Cq method] were calculated and normalized to control - ribosomal *18S RNA* gene expression [7].

The following thermo-cycling conditions were used:

- 50°C for 2 min;
- 95°C for 10 min;
- 40 cycles of 95°C for 15 sec
- 60°C for 60 sec.

Each sample was analyzed in triplicate. All reactions were performed using the ABI PRISM[®] 7900HT Sequence Detection system (Thermo Fisher Scientific, Inc.).

Table 1. Assays analyzed in the study

According to the ^aHUGO gene nomenclature committee and ^bApplied Biosystems Life Technologies

Gene symbol	Gene product name	Gene ID ^a	Assay ID ^b
NOTCH1	notch 1	7881	Hs01062014_m1
HES1	hes family bHLH transcription factor 1	5192	Hs00172878_m1
DLL1	delta like canonical Notch ligand 1	2908	Hs00194509_m1
DLL3	delta like canonical Notch ligand 3	2909	Hs01085096_m1
DLL4	delta like canonical Notch ligand 4	2910	Hs00184092_m1
JAG1	jagged 1	6188	Hs01070032_m1
JAG2	jagged2	6189	Hs00171432_m1
18SRNA	ribosomal 18SRNA	1383	Hs99999901_s1

RESULTS

As a result of mRNA relative expression analysis of Notch signaling genes in non-small cell lung cancer cell lines (H520, H1299, A549 and Calu-3) and in human bronchial epithelial cells, we obtained NOTCH1 receptor and HES1 target gene to be significantly decreased in all NSCLC lines (Fig. 1).

Moreover, we investigated the mRNA expression of NOTCH1-before and after treatment with GSI-RO4929097 in A549 cells. We observed downregulated mRNA expression of chosen receptor, as well as the slower proliferation of A549 cells after GSI application (Fig. 2).

Furthermore, we measured mRNA level of all Notch ligands, as follow: DLL1, DLL3, DLL4, as well JAG1 and JAG2. The mRNA status of DLL1 was also greatly downregulated, whereas in Calu-3, the mRNA of DLL1 decreased to almost undetectable level (Fig. 3, 4).

Interestingly, DLL3 and DLL4 were upregulated in some NSCLC cells when compared to HBEpC cells. Accordingly, in H1299 and in A549 cells mRNA DLL3 was increased, while expression of DLL4 was elevated in H520 and slightly higher in A549. Nevertheless, other type of Notch ligands – JAG1 and JAG2, were characterized by very low expression at mRNA level in all used NSCLC cell lines.

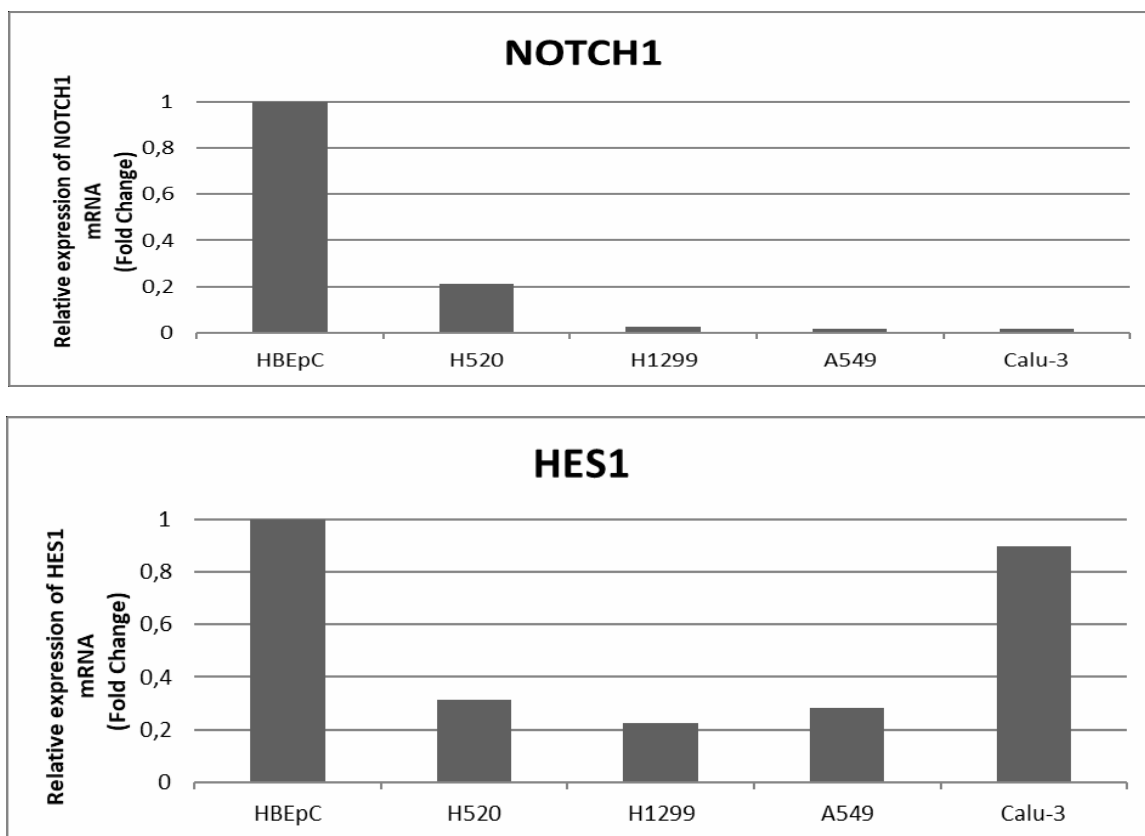


Figure 1. Relative expression of mRNA NOTCH1 and HES1 level in NSCLC cell lines compared to HBEpC cells

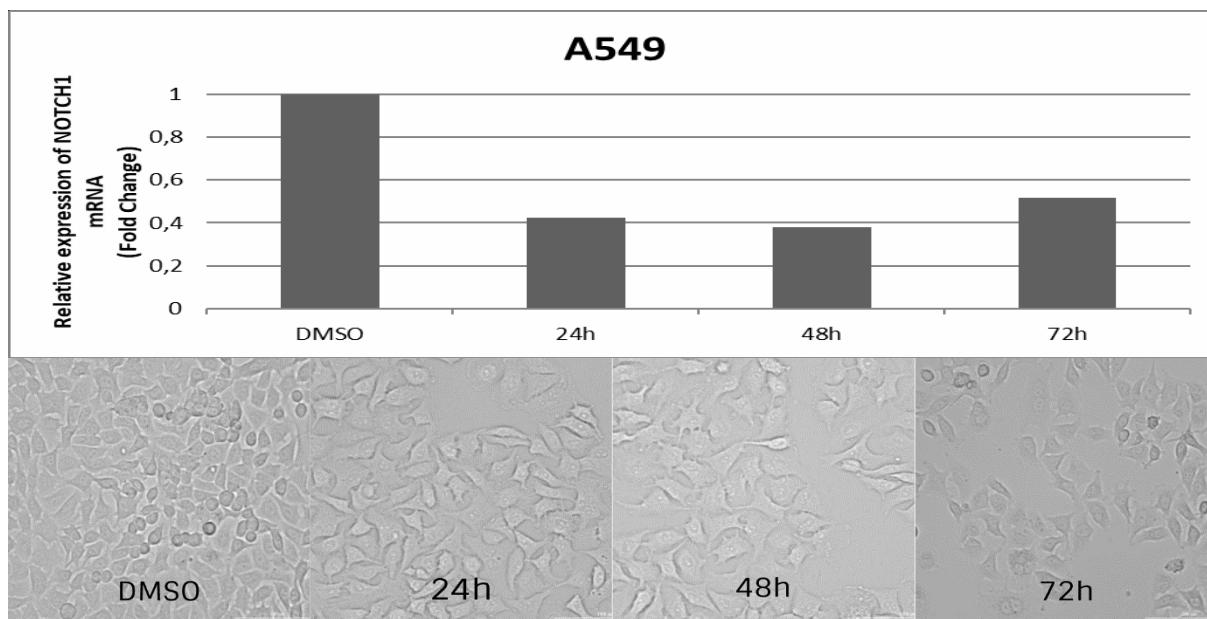


Figure 2. Relative expression of mRNA NOTCH1 in A549 cells before and after 20µM treatment with GSI for 24, 48 and 72h (upper graph) and suppressed proliferation of A549 cells based on kinetic proliferation assay using label free cell counting (lower graph).

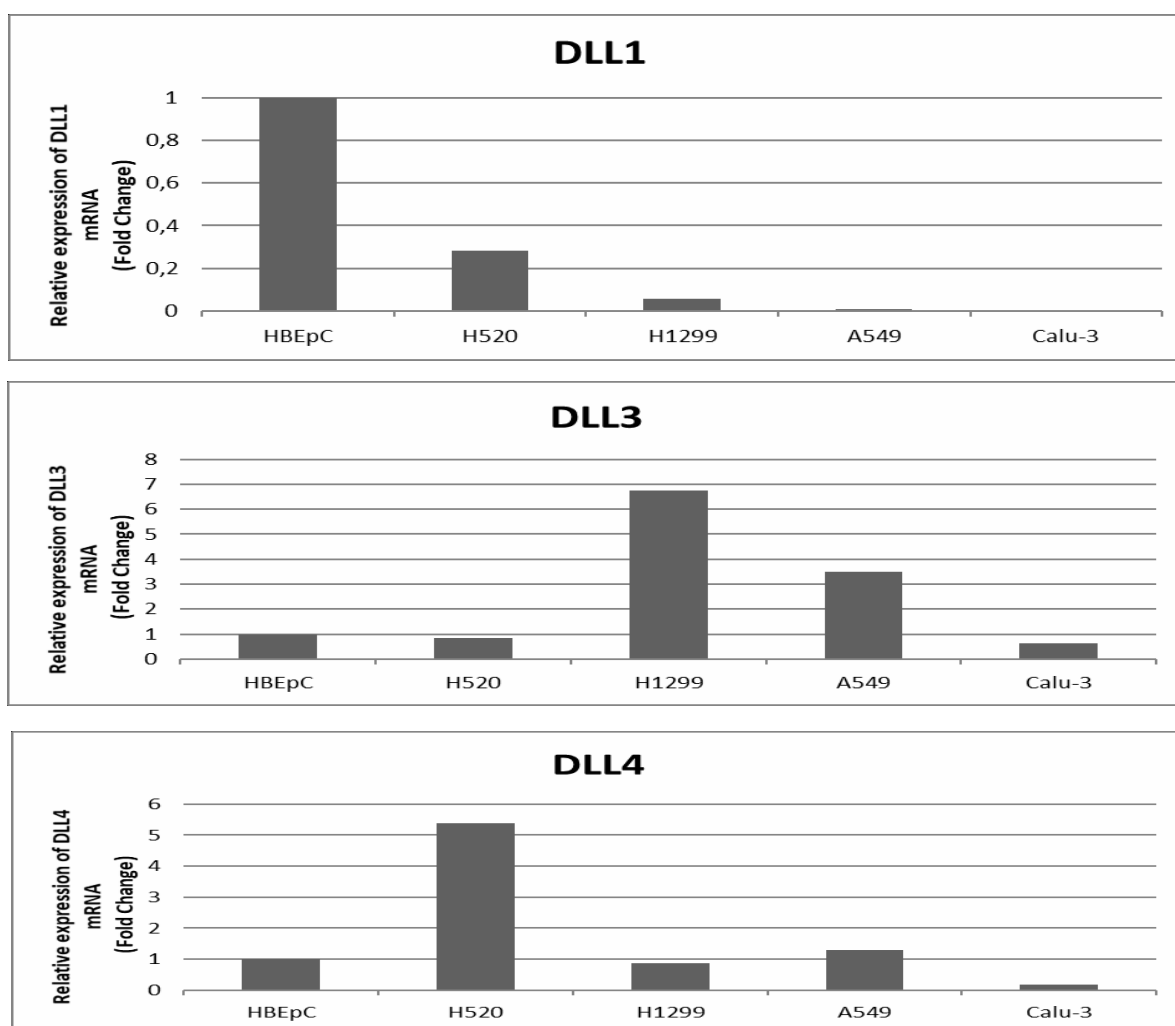


Figure 3. Relative expression of mRNA Dll1, Dll3, Dll4 level in NSCLC cell lines compared to HBEpC cells

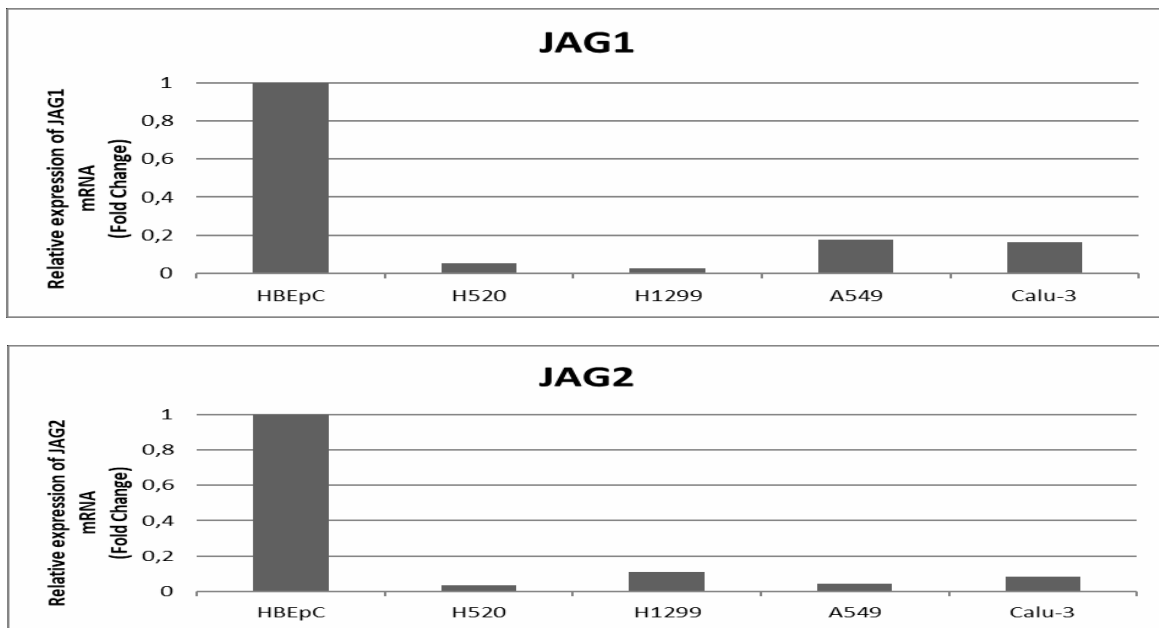


Figure 4. Relative expression of mRNA JAG1 and JAG2 level in NSCLC cell lines compared to HBEpC

DISCUSSION

Notch signaling is associated with many physiological processes in the lung, such as lung development, regulation of cells proliferation and apoptosis [8]. It is also engaged in various aspects of cancer biology, including metastasis, angiogenesis and cancer cells proliferation [9,10,11]. A growing body of evidence indicates that Notch receptors and ligands are characterized by altered mRNA expression in lung cancer cells, but results are contradictory [12]. Nevertheless, most of the researchers agree that NOTCH1 is activated in lung cancer [13,14]. The gamma secretase inhibitors (GSIs) are common drugs which are used to decrease activated form of NOTCH1 influencing cancer cells proliferation and apoptosis [9,14]. Therefore, in our current studies, we intended to confirm GSI effect on A549 cells. As a result, it has been noticed that A549 cells grew slower after incubation with GSI. Moreover, our experiments show that mRNA expression of NOTCH1 decreased after drug implementation. Presented data is consistent with other available *in vivo* and *in vitro* studies in that matter [15,16,17].

Nevertheless, the major problem in evaluating the role of Notch signaling in lung cancer is still actual initial expression of Notch ligands and receptors. Therefore, our further experiments were designed to demonstrate basal expression of Notch compartments, as well as common target gene HES1. We decided to compare mRNA expression from NSCLC cell lines to results obtained in HBEpC cell. Human bronchial epithelial cells (HBEpC) were used as a non-cancerous control line. According to general understanding, bronchial cells are the same

epithelial source as NSCLC cells, therefore they seems to be a good model to study all aspects of lung epithelial function and disease. Moreover, it allows us to see whether we can notice the difference in mRNA status between non-cancerous and cancer cells. In fact, our experiments shows that NOTCH1, HES1 and most of the ligands were downregulated at mRNA level compared to HBEpC. This may be not steady with available studies. Nevertheless, investigators persist in the opinion that Notch signaling expression is tissue dependent and highly correlate with microenvironment. For example, the results of Chen and colleagues show the significance of oxygen level on the biological outcome of Notch signaling overall. The authors indicate that hypoxia may be important in upregulation of Notch signaling in lung cancer; moreover, it can make cancer cells more sensitive to GSI inhibition. This report clearly specifies that cellular context is crucial in Notch expression in NSCLC [18]. It seems probable, that in our experiments the same factors could be involved. However, we have noticed downregulation of NOTCH1 and DLL1, JAG1, JAG2 at mRNA level in all cancer cell lines used in the study. Our results are in accordance with previous findings performed in more than 60 patients' samples where tumor cells showed lower Notch genes expression than non-cancerous tissue derived from the same patient [19]. On the other hand, in the study accomplished in patients' samples by Britta Westhoff at coworkers it has been revealed that Notch pathway is highly active and strongly correlate with overall survival. It has been presented that alterations of Notch pathway are common and include Notch mutations which strictly correlate with Notch activation status [20]. Moreover, in our opinion, there is another

factor that should be taken into account when analyzing Notch signaling, that is correlation with other pathways [21].

In summary, the research presented here shows that Notch signaling expression is altered in NSCLC cell lines, and its inhibition through NOTCH1 may decrease proliferation of A549 cells. Nonetheless, the evaluation of Notch signaling expression should be assessed carefully taking into account factors that affect the mRNA status including proper control [22].

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this study.

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