

# Evaluation of the expression of Notch1 and related proteins in lung carcinoma cells

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## Research article

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# Abstract

Introduction Notch signaling pathway has different roles in many human neoplasms, being either tumor-promoting or anti-proliferative. In addition, Notch signaling in carcinogenesis could be tissue dependent. Aim To study the relation between Notch1 protein expression in lung cancer cells to the following Notch related proteins: Hes1, c-Myc, Jagged1 and Jagged2. Materials and methods Notch1 and its related proteins were detected in human lung cancer cell lines and in 54 surgically resected different lung carcinoma tissues. Then, we used small interfering RNA (siRNA) technology, to down-regulate the expression of Notch1 in H69AR and SBC3 small cell lung carcinoma (SCLC) cells. Also, we transfected venus Notch1 intracellular domain (v.NICD) plasmid into human SCLC lines; H69. Results: The expression of Hes1, c-Myc and Jagged2 is affected by Notch1 in SCLC. Conclusion There is a strong association between the expression of Notch1 protein and the expression of Hes1, c-Myc and Jagged2 proteins, which could aid in better understanding the tumorigenesis in SCLC.

## Background

Lung cancer is classified into two main types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), which is further sub-divided into: adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma [1,2]. SCLC accounts for 20% of lung cancer, and is characterized with low survival rates, frequent recurrence and failure of therapy [3].

Notch pathway is one of the most important cell signaling pathways, which acts through the interaction with ligands of the Delta (DLL1, DLL3 and DLL4) and Jagged (Jagged1 and Jagged2) family, leading to the proteolytic cleavage of Notch receptor, releasing the Notch intracellular domain (NICD) into the cytoplasm, which enters the nucleus, and induces the transcription of several genes; Hes1, cyclin D1, c-myc, Akt and others [4].

Notch signaling in tumorigenesis can be either oncogenic or anti-proliferative. In lung carcinoma, we previously showed that Notch1 signaling is suppressed in SCLC, by histone deacetylation around the promoter region of *Notch1* [5] and the restoration of Notch1 expression in SCLC leads to the concurrent appearance of epithelial-like areas within the SCLC, and overexpression of Notch1 resulted in inhibition of SCLC growth and could play a role in cell chemo-resistance [5-8]. Moreover, we showed that in NSCLC, Notch1 expression has a tumor inhibitory effect on ADC cells, but not SCC cells [6]. The present study investigates the possible related proteins to Notch1 signaling, aiming for better understanding of Notch1 pathway in lung carcinoma cells.

## Methods

### Cell lines

Human lung cancer cell lines were purchased from American Type Cell Collection (Rockville, MD): H69, H69AR, H889, and H1668 (SCLC), H358 and H1975 (ADC) and H226 and H2170 (SCC). SBC3 cell line was

a gift from Dr.Makato Suzuki (Department of Respiratory Surgery, Graduate School of Medical Sciences, Kumamoto University). A549 ADC cell line was afforded by RIKEN Bio Resource Center (Tsukuba, Japan). Growth media were purchased from Wako Pure Chemical Industries (Ltd., Osaka, Japan). All cells were cultured as previously described [6].

### **Transfection with siRNA**

H69AR and SBC3 cells were used in this experiment. The cells were grown and transfected with Notch1 specific siRNA and RNAi Negative control (Invitrogen, Carlsbad, CA) using Lipofectamine RNAi MAX (Invitrogen); as described in manufacture's instruction. The sequences for siRNA were as follows: for Notch1, sense strand 5'- UCG CAU UGA CCA UUC AAA CUG GUGG-3', antisense strand 5'-CCA CCA GUU UGA AUG GUC AAU GCGA-3'. Stable lines were cloned and harvested at 48 h post-transfection.

### **Construction of recombinant plasmid and transfection**

A recombinant plasmid bearing v.NICD gene (CMV-activated Notch1-venus-pA, generous gift from Dr.Mitsuru Morimoto; Laboratory of Lung Development and Regeneration, RIKEN center for Developmental Biology, Kobe, Japan) and control plasmid eukaryotic expression vector (PcDNA3.1-EGFP, Invitrogen) were prepared as previously described [6]. QIAGEN Plasmid Midi Kit was used to extract the plasmid, as described in manufacture's instruction. H69 cells were used for transfection with plasmids using Lipofectamine LTX (Invitrogen) as described in manufacture's instruction. Stably transfected resistant cell lines were cloned as previously described [6].

### **Western blotting (WB) analysis**

Cells were prepared for WB as previously described [6]. List of primary antibodies used are listed in table 1. The membrane was then incubated with the appropriate secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the immune complex was visualized with the ECL system (Santa Cruz, Texas, US).

### **Immunofluorescence (IF)**

Cells were plated in 24 well plates and were treated as previously described [6]. List of primary antibodies used are listed in table 1. Cells were incubated with the appropriate secondary antibodies (Alexa Flour, Molecular Probes,Eugene, OR) and examined by fluorescent microscope (Olympus, Tokyo, Japan).

### **Histopathological evaluation**

Tissue samples of lung ADC (n=31), SCC (n=9) and SCLC (n=14) were obtained from anonymous cases of lung cancer patients, who were surgically treated at Kumamoto University Hospital. All samples were fixed in 10% formalin and embedded in paraffin. Tissue sets were stained with hematoxylin and eosin (H&E) staining and additional sections were used for immunohistochemical (IHC) staining; as previously described [6]. The list of primary antibodies used are listed in table 1. The appropriate secondary antibody

(Envision+System-HRP Labelled Polymer, Dako, Glostrup, Denmark). All slides were examined twice, by the researcher and another independent pathologist in a blinded fashion. The localization of Notch1 and its related proteins (NICD, Hes1 and Jagged1) was observed. A semi-quantitative method to assess the staining intensity was used: a strong positive result was defined as strong immunoreactivity in 50% or more of tumor cells, a weak positive result was defined as weak immunoreactivity or staining of fewer than 50% of tumor cells, and tumors with no or minimal staining were scored as negative.

## Results

### Expression of Notch1 and related proteins in lung cancer (Fig.1)

By WB, Notch1 and c- Myc were detected in NSCLC cells. In contrast, all SCLC cells-except H69AR and SBC3- lacked Notch1, but weakly expressed c-Myc, as did H1688 cells. Hes1 and Jagged1 were expressed in both SCLC and NSCLC cells (**Fig.1a**). To detect cellular localization of Notch1 and its related proteins, we performed IF for selected cell lines: H69 and H1688 (SCLC not expressing Notch1), H69AR and SBC3 (SCLC expressing Notch1), A549 and H2170 (NSCLC). In H69 and H1688 cells, Hes1 was detected in mainly in cells nuclei and occasionally in cytosol, while Jagged 1 was seen mainly within cytosol. In the rest of cells expressing Notch1, Hes1 and Jagged1 were detected mainly in cytosol (**Fig.1b**). To confirm our *in vitro* findings, IHC staining of human lung carcinoma tissue was done. In SCLC, Notch1 was absent, Hes1 was weakly positive in nuclei and Jagged1 was strongly positive in nuclei. In NSCLC, Notch1 and NICD were strongly positive in ADC, while weakly positive in SCC. Hes1 was weakly expressed mainly in cytoplasm of cells, while Jagged1 was strongly positive mainly in cytosol of cells (**Fig.1c**).

### Knocking down (KD) Notch1 and transfection of Notch1 versus NICD (v.NICD) plasmid

WB, IFA and RT-PCR were used to detect the efficacy of siRNA against Notch1 in transfected cells and to ensure v.NICD transfection into H69 cells (**Fig.2**).

### Effect of Notch1 KD and overexpression on Notch related proteins (Hes1, c-Myc, Jagged1 and Jagged2) (Fig. 2)

In SCLC, Hes1 and c-Myc protein expressions were decreased in cells with KD Notch1 and increased in H69 cells transfected with v.NICD plasmid. In NSCLC, neither Hes1 nor c-Myc protein expressions were affected by KD Notch1. Regarding Jagged1, its expression wasn't affected by either KD or induction of Notch1. However, Jagged2 expression was increased in SCLC cells with KD Notch1; especially H69AR cells and in H69 cells transfected with v.NICD. Moreover, Jagged2 expression was decreased in NSCLC cells with KD Notch1, especially A549 cells.

## Discussion

Despite rapidly accumulating information, the role of Notch signaling in oncogenesis is far from fully understood, due to the complex nature of Notch signaling and that differences in cell type could affect its final outcome [9]. Our present report focuses on the relation between Notch1 and Notch related proteins in SCLC and NSCLC cells.

Our data showed that Notch1 receptor and its related proteins (Hes1, c-myc and Jagged1) were significantly overexpressed in NSCLC and not in SCLC, consistent with other's observations [10-12]. In SCLC, we detected also expression of Hes1 and Jagged1 proteins despite the absence of Notch expression, while no expression of c-Myc protein was detected, except in H69AR and SBC-3-both expressing Notch1- and H1688 cells, which don't express Notch1. Such Hes1 protein expression in SCLC that do not express Notch 1 can be explained by the fact that other signaling pathways control Hes1 protein expression, as EGFR and FGFR [13]. Regarding c-Myc, its expression in SCLC cell lines has been reported in the variant class of SCLC; that is characterized by adherent cell growth and morphology similar to undifferentiated LCC [14, 15]. In our study, we used the following SCLC cells: H69, H889, H69AR, H1668 and SBC-3, all of which were of the classic type, despite the adherent growth pattern of the latter three. H69 cells showed weak c-Myc expression. H69AR and H1688 cells cell lines showed c-Myc expression, as previously reported [16-18]. Regarding SBC-3 cells, this is the first report about c-Myc protein expression in them. These observations suggest that c-Myc might be linked to Notch1 expression- especially in SCLC- as we further proved in our study.

In addition, we demonstrated that Hes1 and Jagged1 were detected in the nuclei of SCLC cells. There may be some undetected mechanisms for Hes1 to go inside the nuclei from the cytosol, as suggested by previous studies [19, 20]. On the other hand, nuclear localization of Jagged1 can be explained by the fact Jagged1 has an intra-cellular domain, which contains nuclear localization signals that permit their entry into the nucleus [21]. Regarding NSCLC, cellular localization of both Hes1 and Jagged1 show differences between detecting them in cell lines compared to tissue sections. In cell lines, both proteins were seen in cytosol of NSCLC cells. In tissue sections, both proteins were detected in cytosol and nuclei of ADC cells, while they were mainly seen in nuclei of SCC cells. This could be attributes to the antibodies used or to the difference in cell biological behavior when grown in cell lines or in tissues. We believe that clarifying the mechanism of Notch1 related proteins subcellular trafficking may give an additional insight for better understanding the role of Notch1 signaling in lung carcinoma.

By utilizing siRNA analysis, we demonstrated the effect of KD of Notch1 in H69AR and SBC-3 cells and confirmed such effect by observing the results of overexpressing Notch1 in H69 cells. Moreover, we showed the effect of KD Notch1 in NSCLC cells; A549 and H2170 cells.

We found that KD Notch1 decreased Hes1 and c-Myc expression in transfected H69AR and SBC-3 cells, and that expression of Notch1 in H69 cells increased their expression. This indicates close interaction between Notch1 with Hes1 in SCLC, as previously reported [22,23]. In addition, our results suggest that c-Myc is a downstream molecule of Notch1 signaling in SCLC cells, as previously reported in T cell acute lymphoblastic leukemia [24-26]. In NSCLC cells, we couldn't observe any effect of KD Notch1 on Hes1 or

c-Myc expressions, suggesting that these two proteins are not related to Notch1 signaling in NSCLC cells. Moreover, we found that Notch1 affect Jagged2 expression-in both SCLC and NSCLC cells- while has no effect on the expression of Jagged1. This can be explained by the fact that Jagged1 is associated with Notch3 signaling in lung carcinoma-as we previously reported- [27], and in other carcinomas; ovarian carcinoma, pancreatic cancer and cervical SCC [28-31]. Moreover, our results confirm the fact that Jagged1 and Jagged2 have different biological roles as previously stated [32].

In comparison to Notch3 signaling, we showed that -in both NSCLC and SCLC cells- Jagged1 and Hes1 expressions were affected by Notch3 protein, and that Nocth1 protein expression was decreased by KD *Notch3* in H69AR cells, indicating a close interaction between both Notch1 and Notch3 signaling in SCLC cells [27].

## Conclusion

Conclusion Notch1 signaling plays an important role in lung carcinogenesis. Specific Notch1 target genes and ligands were identified in the present study, yet the complex nature of the Notch signaling in tumorigenesis is still complicated and identification of other Notch signaling components is necessary for better understanding of the role of this signaling in lung cancer.

## Declarations

### Availability of data and materials

Data are available from the corresponding author upon a reasonable request.

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### **Contribution**

This study was performed under the supervision of Prof. TI; Head of the department of pathology and experimental medicine; Kumamoto University. WH contributed to the conception and design of the study. TI contributed to the acquisition of the data. WH and TI contributed to the analysis, interpretation and revising the data. TI had access to the final version of the manuscript and approved the version to be published. All authors have read and approved the manuscript

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### **Ethics Declarations**

#### **Ethics approval and consent to participate**

The study followed the guidelines of the Ethics Committee of Kumamoto University. Informed consent was waived because of the retrospective design of the study, and the information of each patient was anonymized prior to analyses.

#### **Consent for publication**

Not applicable.

#### **Competing interests**

The authors declare that they have no competing interests.

## **Abbreviations**

**ADC:** Adenocarcinoma (ADC)

**Hes1:** Hairy and enhancer of split-1.

**IF:** Immunofluorescence

**IHC:** Immunohistochemistry

**KD:** Knocking down (KD)

**NICD:** Notch intracellular domain (NICD)

**NSCLC:** Non-small cell lung carcinoma (NSCLC),

**SCC:** Squamous cell carcinoma (SCC)

**SCLC:** Small cell lung carcinoma (SCLC)

**siRNA:** Small interfering RNA (siRNA)

**v.NICD:** venus Notch1 intracellular domain (v.NICD)

**WB:** Western blot

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## Tables

**Table 1:** Antibodies for western blot, immunofluorescence and immunocytochemistry. References, quantities and working dilutions are indicated.

Primary antibodies	Reference	Lot	WB	IF	IHC
Rabbit anti-Notch1	CS, Danvers, MA	C44H11	1:500		
	CS	D1E11			1:200
	SC, Santa Cruz, CA	H-131, 9170		1:50	
Rabbit anti-Notch1 NICD	CS	Val1744, D3B8,	1:500		
Rabbit anti-Hes1	Gifted from T. Sudo, TORAY Industries, Yokohama		1:10,000		
	Abcam, Cambridge, UK	ab49170		1:100	1:200
Rabbit anti-Jagged1	Epitomics, Burlingame, California	ab3772-1	1:10,000	1:200	1:400
Rabbit anti-Jagged2	CS	C83A8	1:5000		
Rabbit anti-c-Myc	CS	D84C12, XP™	1:1000	1:800	
Mouse anti-β actin	Sigma Aldrich, Ontario, Canada	AC-15	1:20,000		

**Abbreviations:** IF: immunofluorescence, WB: western blot, IHC: immunohistochemistry, CS: Cell signaling, SC: Santa Cruz Biotechnology, NICD: Notch Intracellular domain, Hes1: hairy and enhancer of split-1.