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# Identifying specific *Notch1* target proteins in lung carcinoma cells

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**Summary.** Background. The Notch signaling pathway has different roles in many human neoplasms, being either tumor-promoting or anti-proliferative. In addition, Notch signaling in carcinogenesis can be tissue dependent. The aim of the current study is to elucidate the relation between Notch1 protein expression in lung cancer cells and the following Notch related proteins: Hes1, c-Myc, Jagged1 and Jagged2.

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 tumorigenesis in SCLC.

**Key words:** Human lung cancer, Notch1 signaling, Small interfering RNA (siRNA), Venus Notch1 intracellular domain (v.NICD)

## Introduction

### *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 (Bunn, 2012; Travis et al., 2015). SCLC accounts for 20% of lung cancer, and is characterized with low survival rates, frequent recurrence and failure of therapy (Fischer and Arcaro, 2008).

The 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 (Rizzo et al., 2008).

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* (Hassan et al., 2017) 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 (Wael et al., 2014; Hassan et al., 2014, 2016a,b, 2017). Moreover, we showed that in NSCLC, Notch1 expression has a tumor inhibitory effect on ADC cells, but not SCC cells (Wael et al., 2014). The present study investigates the possible related proteins to Notch1 signaling, aiming for better understanding of Notch1 pathway in lung carcinoma cells.

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**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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## Materials and methods

### *Cell lines*

Human lung cancer cell lines were purchased from American Type Cell Collection (Rockville, MD): H69, H69AR, H889, and HI668 (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 (Wael et al., 2014).

### *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 the manufacturer's instructions. 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 (Wael et al., 2014). QIAGEN Plasmid Midi Kit was used to extract the plasmid, as described in the manufacture's instructions. H69 cells were used for transfection with plasmids using Lipofictamine LTX (Invitrogen) as described in manufacture's instruction. Stably transfected resistant cell lines were cloned as previously described (Wael et al., 2014).

### *Western blotting (WB) analysis*

Cells were prepared for WB as previously described (Wael et al., 2014). 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 (Wael et al., 2014). 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) and additional sections were used for immunohistochemical (IHC) staining; as previously described (Wael et al., 2014). The primary antibodies used are listed in table 1. 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 Jagges1) 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

**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 | | |

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.

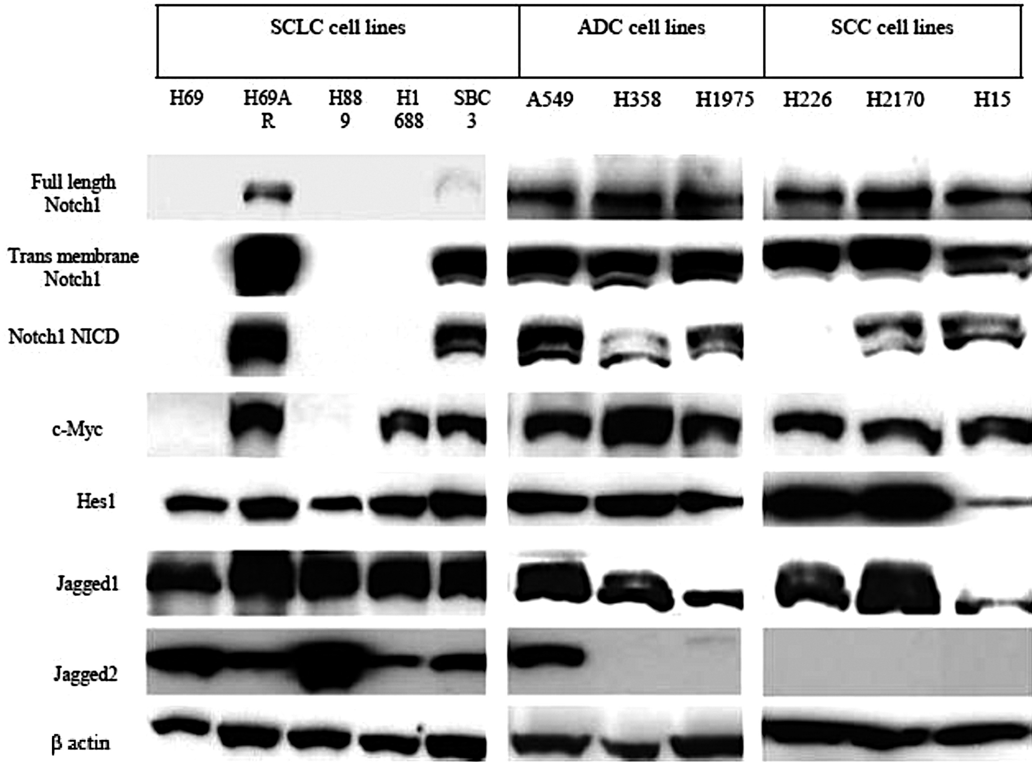


**Fig. 1.** Expression of Notch1 and its related proteins (c-Myc, Hes1, Jagged1 and jagged 2) in human lung cancer cells. Notch1 protein expression in human small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines; adenocarcinoma (ADC) and squamous cell carcinoma (SCC). Cells cultured in the same conditions were supplied for western blotting (WB). The expression of β-actin was used as an internal control. Notch1 (transmembrane part and intra-cellular domain (NICD) was mainly detected in NSCLC cells, and in H69AR and SBC-3 SCLC cells. Hes1 was detected in all lung cancer cells. c-Myc was detected mainly in NSCLC cells, and in H69AR, H1688 and SBC-3 SCLC cells. H69 cells show weak c-Myc expression. Jagged1 was expressed in both SCLC and NSCLC cells. The experiment was performed in triplicate.

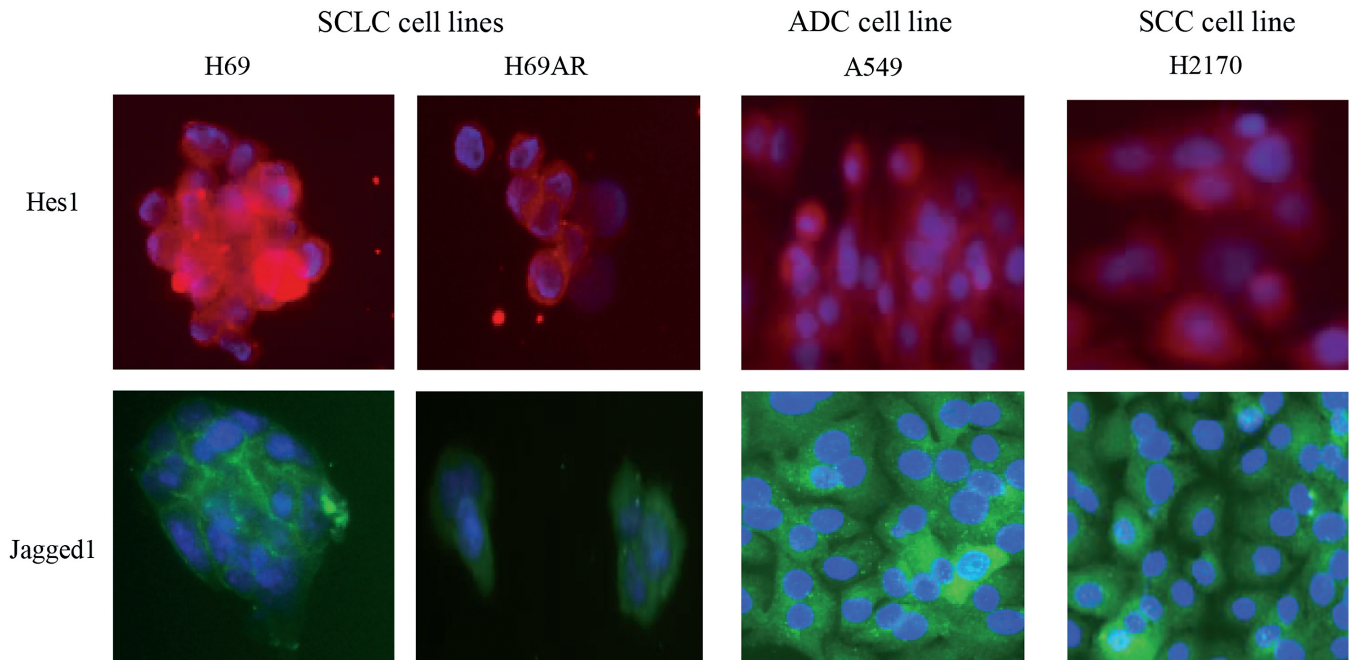


**Fig. 2.** Expression of Notch1 and its related proteins (Hes1 and Jagged1) in human lung cancer cells. Representative photos of immunofluorescence (IF) analysis for difference in expression of Hes1 and Jagged 1 in SCLC and NSCLC cells. In H69, Hes1 was detected mainly in cells nuclei, while Jagged 1 was seen mainly within cytosol. In Notch1 expressing cells (H69AR and NSCLC), Hes1 and Jagged1 were detected mainly in cytosol. Hes; hairy and enhancer of split.

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 (Figs. 1-3)*

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. 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 mainly in cell 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. 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 negative in a majority of cases of both ADC and SCC. In ADC cases with Hes1 protein expression (n=5), it was strongly expressed in nuclei and cytoplasm of tumor cells, while its expression in SCC cases (n=2) was detected in nuclei of tumor cells. Similarly, Jagged1 was detected in few cases of ADC (n=7) with both nuclear and cytoplasmic strong localization, while in SCC few cases (n=3) Jagged 1 was weakly expressed in nuclei of tumor cells (Table 2).

### *Knocking down (KD) Notch1 and transfection of Notch1 venus 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. 4).

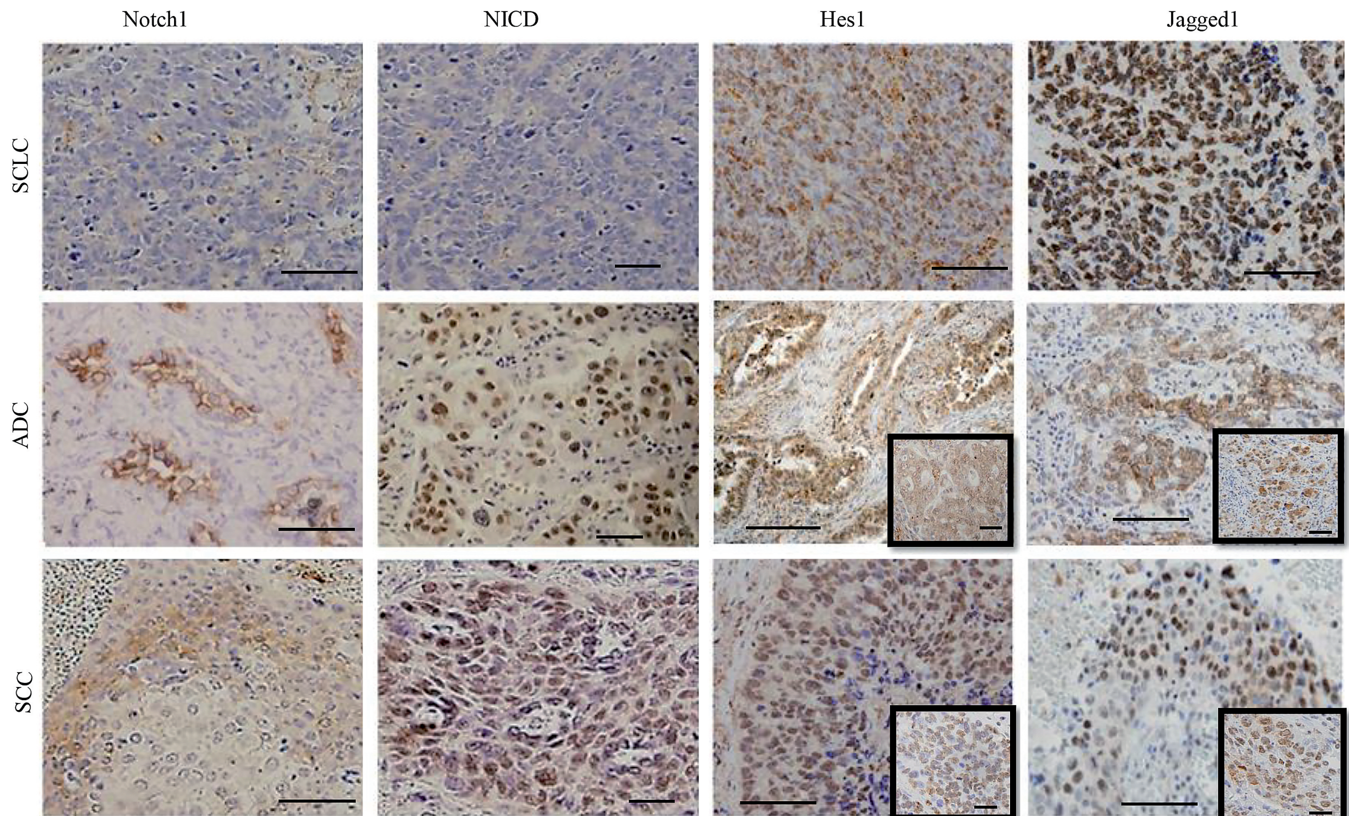


**Fig. 3.** Expression of Notch1 and its related proteins (Hes1 and Jagged1) in human lung cancer tissue sections. Representative photos for immunohistochemical (IHC) staining of Notch1, NICD, Hes1 and Jagged 1 in different lung cancer tissues. The cytoplasmic and membranous immunoreactions (brown) for Notch1 and nuclear expression of NICD were evident in NSCLC cells; especially ADC, while absent in SCLC cells. Hes1 and Jagged 1 were detected in SCLC nuclei. In NSCLC, Hes1 and jagged1 were expressed in both nuclei and cytoplasm of ADC cells and mainly in nuclei of SCC cells. Hes; hairy and enhancer of split. Scale bars: NICD, 50 $\mu$m; rest of figures, 100 $\mu$m; insets, 20 $\mu$m.

### *Effect of Notch1 KD and overexpression on Notch related proteins (Hes1, c-Myc, Jagged1 and Jagged2) (Fig. 4)*

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 was not 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 outcome (Artavanis et al., 1999). 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 (Robinson et al., 1995; Chen et al., 1997; Li et al., 2010). In SCLC, we detected also expression of Hes1 and Jagged1 proteins despite the absence of Notch expression. Such Hes1 protein expression in SCLC cells that do not express Notch 1 can be explained by the fact that other signaling pathways control Hes1 protein expression, such as EGFR and FGFR (Liu et al., 2015). 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 (Little et al., 1983; Nasgashio et al., 2011). In our study, we used the following SCLC cells: H69, H889, H69AR, HI668 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 cell lines showed c-Myc expression, as previously reported (Doyle et al., 1996; Barsyte-

**Table 2.** Results of immunohistochemical staining of human lung carcinoma in surgically resected samples.

| Tumor type/ Protein expression | SCLC (n=14) | NSCLC (n=40) | |
|---|---|---|---|
| | | ADC (n=31) | SCC (n=9) |
| Notch1 | Negative (n=14) | Strong positive (n=31) | Weak positive (n=9) |
| NICD | Negative (n=14) | Strong positive (n=31) | Weak positive (n=9) |
| Hes1 | Weak positive in nuclei (n=12) | Negative in most cases (n=26). Strongly positive in nuclei and cytoplasm in few cases (n=5) | Negative in most cases (n=7). Weakly positive in nuclei in few cases (n=2) |
| Jagged1 | Strong positive in nuclei (n=10) | Strongly positive in nuclei and cytoplasm in few cases (n=7) | Negative in most cases (n=6). Weakly positive in nuclei in few cases (n=3) |

SCLC, Small cell lung carcinoma; NSCLC, Non-small cell lung carcinoma; ADC, Adenocarcinoma; SCC, Squamous cell carcinoma; NICD, Notch Intracellular domain; Hes1, hairy and enhancer of split-1.

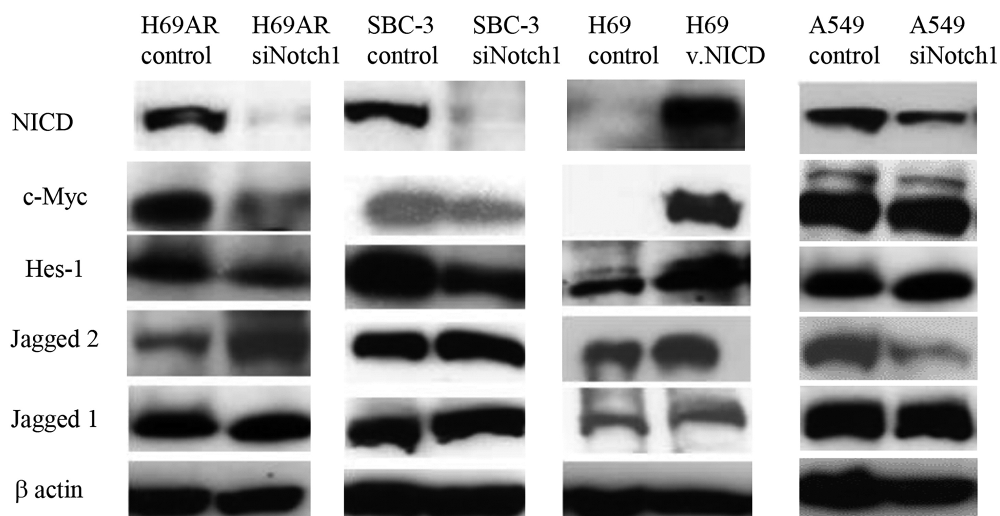


**Fig. 4.** Effect of Notch1 on Notch related proteins (c-Myc, Hes1, Jagged1 and Jagged2). WB of NICD and other Notch-related proteins (c-Myc, Hes1, Jagged1 and Jagged2) 48 h after siRNA transfection and after stable v.NICD plasmid transfection. In SCLC, Hes1 and c-Myc protein expressions were decreased in cells with KD Notch1 and increased in cells with induction of Notch1. Regarding Jagged1, its expression was not affected by Notch1. However, Jagged2 expression was increased in SCLC cells with KD Notch1; especially H69AR cells and H69 cells transfected with v.NICD. The expression of β -actin was used as an internal control. The experiment was performed in triplicate. Hes; hairy and enhancer of split.

Lovejoy et al., 2006; Olejniczak et al., 2007). 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. However, the expression of c-Myc in H1688 cells without concomitant Notch1 protein expression could indicate that other signaling pathways affect c-Myc signaling in SCLC cells.

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 (Kamakura et al., 2004; Zheng et al., 2008). On the other hand, nuclear localization of Jagged1 can be explained by the fact that Jagged1 has an intra-cellular domain, which contains nuclear localization signals that permit their entry into the nucleus (Urs et al., 2008). 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 only some cases of ADC tumor cells (16-22%), while they were mainly seen in nuclei of some cases (22-33%) of SCC tumor cells. This could be attributed 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 an 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 (Shan et al., 2007; Kuramoto et al., 2012; Fujino et al., 2015). 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 (Palomero et al., 2006; Weng et al., 2006; Sharma et al., 2007). In NSCLC cells, we could not 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, although it has been reported that Myc cooperated with Notch signaling in generation of lung ADC (Zou et al., 2018). Moreover, we found that Notch1 affects Jagged2 expression-in both SCLC and NSCLC cells- while it 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- (Hassan et al., 2017), and in other carcinomas; ovarian carcinoma, pancreatic cancer and cervical SCC (Choi et al., 2008; Chen et al., 2010; Yeasmin et al., 2010; Vo et al., 2011). Moreover, our results confirm the fact that Jagged1 and Jagged2 have different biological roles as previously stated (Osanyingbemi-Obidi et al., 2011).

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 (Hassan et al., 2016b).

## 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 tumorgenesis is still complicated and identification of other Notch signaling components is necessary for better understanding of the role of this signaling in lung cancer.

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*Contribution.* 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. Both authors have read and approved the manuscript

*Ethics approval and consent to participate.* The study was approved by The Ethics Committee of Kumamoto University (Research approval number #342). 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.

*Availability of data and materials.* Data are available from the corresponding author upon reasonable request.

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