Evaluation of the Expression of Notch1 and Related Proteins in Lung Carcinoma Cells

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Abstract

Introduction: Notch signaling pathway has different roles in many human neoplasms, including lung carcinoma; both small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Notch1 signaling could be either tumor-promoting or anti-proliferative depending on cellular context. Aim: To study the relation between Notch1 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 and in A549 adenocarcinoma cells. Also, we transfected venus Notch1 intracellular domain (v-NICD) plasmid into H69 SCLC cells.

Results: Notch1 is mainly detected in NSCLC cells. The expression of Hes1, c-Myc and Jagged2 is affected by Notch1 mainly in SCLC cells. Cellular localization of Hes1 and jagged1 proteins could be related to Notch1 expression.

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 lung carcinoma cells.

Keywords: Human lung cancer; Small Cell Lung Carcinoma (SCLC); Non-Small Cell Lung Carcinoma (NSCLC); Notch1 signaling; Notch1 related proteins

Abbreviations: IF: Immunofluorescence, WB: Western Blot, IHC: Immunohistochemistry, CS: Cell Signaling, SC: Santa Cruz Biotechnology, NICD: Notch1 Intracellular Domain, Hes1: Hairy and Enhancer of Split-1

Introduction

Lung cancer is classified into two main types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), which is further divided into: Adenocarcinoma (ADC) – the most common, 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 mechanisms of cell signaling, which acts through the interaction with ligands of the Delta (DLL1, DLL3 and DLL4) and/or Jagged/Serrate (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 a tumorigenesis can be either oncogenic or anti-proliferative. In lung carcinoma, we previously showed that Notch1 signaling is suppressed in SCLC, by an epigenetic mechanism; 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 a cell chemoresistance [6-8]. 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 receptor, aiming for better understanding of Notch1 signaling and its role in lung carcinoma cells.

Materials and 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 (adenocarcinoma; ADC) and H226 and H2170 (squamous cell carcinoma; 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 StealthTM
RNAi Negative control (Invitrogen, Carlsbad, CA) using Lipofectamine RNAi MAX (Invitrogen) as described in manufacturer’s instruction. The sequences for siRNA were as following: for Notch1, sense strand 5'-UCC GUA UGA CCA UUC AAA CUU GUGG-3'; antisense strand 5’-CCA CGA GUU UGA AUG GUC AAU GGGA-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 a 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 H&E staining and additional sections were used for immunohistochemical (IHC) staining; as previously described [6]. The following primary antibodies were used: Notch1, NICD, Hes-1 and Jagged1, as indicated in Table 1. The appropriate secondary antibody (Envision+System-HRP Labelled Polymer, Dako, Glostrup, Denmark) was then applied, followed by visualization with the Liquid DAB+Substrate Chromogen System (Dako). 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**

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 in addition to H1688 cells. Hes1 and Jagged1 were detected in both SCLC and NSCLC cells (Figures 1A-1C). 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 cells nuclei and occasionally in cytosol, while Jagged1 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 weakly expressed mainly in cytoplasm of ADC cells, while stronger expression in SCC cells nuclei was noted. Additionally, Jagged1 was strongly positive in cytoplasm of ADC cells and mainly in nuclei of SCC cells (Figure 1).

**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.

**Effect of Notch1 on Notch related proteins (Hes1, c-Myc, Jagged1 and Jagged2)**

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

<table>
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<th>Primary antibodies</th>
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<th>Lot</th>
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<th>IF</th>
<th>IHC</th>
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<tr>
<td>Rabbit anti-NICD</td>
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<td>--</td>
<td>1:50</td>
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<tr>
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**Table 1:** Antibodies for western blot, immunofluorescence and immunocytochemistry. References, quantities and working dilutions are indicated.
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 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 (Figure 2).

**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 difference 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...
Figure 2: Effect of Notch1 on Notch related proteins (c-Myc, Hes1, Jagged1 and Jagged2).

Note: WB of Notch1-related proteins (c-Myc, Hes1, Jagged1 and Jagged2) 48 h after 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 wasn’t 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.

in SCLC can be explained by a previous study, which detected Hes1 mRNA in SCLC [13]. Regarding c-Myc, its expression in SCLC cell lines is 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, H1688 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 tissue, the expression of Hes1 was weaker when compared to SCLC and cellular localization of both Hes1 and Jagged1 was mainly in cytosol of ADC cells, while in nuclei of SCC cells. 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 expressing 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 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, we showed that in both NSCLC and SCLC cells, Jagged1 and Hes1 expressions were affected by Notch3, and that Notch1 was decreased by KD Notch3 in H69AR cells, indicating a close interaction between both Notch1 and Notc3 signaling in SCLC cells [27-32].

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.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgement
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References