Prognostic significance of Notch ligands in patients with non-small cell lung cancer

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Abstract. The Notch signaling pathway is deregulated in numerous solid types of cancer including non-small cell lung cancer (NSCLC). However, the profile of Notch ligand expression remains unclear. Therefore, the present study aimed to determine the profile of Notch ligands in NSCLC patients and to investigate whether quantitative assessment of Notch ligand expression may have prognostic significance in NSCLC patients. The study was performed in 61 pairs of tumor and matched unaffected lung tissue specimens obtained from patients with various stages of NSCLC, which were analyzed by reverse transcription-polymerase chain reaction. The marked expression levels of certain analyzed genes were detected in NSCLC samples and in noncancerous lung samples. Of the five Notch ligands, jagged 1 (*Jag1*), jagged 2, delta-like protein 1 and delta-like protein 4 were expressed in the majority of tissues, but their expression levels were reduced in NSCLC when compared with noncancerous lung tissue ($P<0.001$). Delta-like protein 3 expression was consistently low and was observed only in 21/61 tumor tissue samples. Taken together, Notch ligands are expressed in NSCLC. However, the expression level is reduced when compared to noncancerous tissue. Furthermore, the present study revealed that quantitative assessment of *Jag1* expression in NSCLC may improve prognostication of patient survival.

Introduction

Lung cancer remains the most common cause of cancer mortality worldwide (1,2). Approximately 80% of all lung cancer patients are diagnosed with non-small-cell lung cancer (NSCLC) (3). Currently, lung cancer therapy is mainly based on Tumor-Node-Metastasis (TNM) disease staging and tumor histological classification. However, despite progress in surgical techniques, chemotherapy and radiotherapy, the 5-year survival rate of patients with lung cancer remains low (~16%) (4,5). Therefore, there is a continuous need to identify specific and sensitive biomarkers that may improve cancer patient management. Such markers should allow prediction and prognostication of patient survival, disease free survival or treatment response (6). Therefore, the current study aimed to investigate potential molecular markers which may become novel prognostic factors in NSCLC, specifically jagged 1 (Jag1), jagged 2 (Jag2), delta-like protein 1 (Dll1), delta-like protein 3 (Dll3), delta-like protein 4 (Dll4), Notch 1 and hairy and enhancer of split-1 (Hes1). The present study focused on the Notch signaling pathway, which is known to have a significant role in tumorigenesis and cancer progression (7,8). The Notch family consists of four receptors (Notch 1-4) and five ligands (Jag1, Jag2, Dll1, Dll3 and Dll4) (9). Notably, receptors and ligands are typically presented on neighboring cells; therefore, ligand binding is triggered via direct cell-cell communication (10). Notch ligands function as Notch signaling agonists, exerting their actions through intercellular interactions (11). However, in mammals, binding between Notch ligands and Notch receptors remains a non-selective process (12). Recent data revealed that certain Notch ligands may be highly expressed in lung cancer cells (5). Furthermore, it has long been known that the lung constitutes the richest source of Notch ligand and receptor mRNA (13). However, to the best of our knowledge, to date the role of Notch ligands in cancer pathogenesis remains to be fully elucidated. Notably, a low rate of mutations observed in Notch ligands in cancer may make them a good target for research on cancer therapy concepts (14). However, despite available knowledge, there remains limited information on the level of Notch ligand expression in NSCLC patients (15-17).

Materials and methods

Patients and tissue samples. The present study was performed on 61 pairs of tumor and matched unaffected lung tissue

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specimens obtained from patients with various stages of NSCLC, aged from 39.8 to 78.1 years (mean, 62.5 years; standard deviation, 8.4 years) who underwent a curative surgery between March 2003 and October 2009 at the Department of Thoracic Surgery, Bialystok Medical University Hospital (Poland). Detailed patient characteristics are presented in Table I.

The samples were collected upon obtaining informed consent from the patients at the time of surgery, and the present study was approved by the Ethics Committee of the Medical University of Bialystok (Bialystok, Poland). Tissue samples were processed immediately following surgical removal. Tumor tissue and unaffected lung tissue specimens from the same lobe or lung of the patient were snap-frozen in liquid nitrogen, followed by storage at -80˚C. Prior to processing, the sections of frozen tissue specimens were stained with hematoxylin and eosin and evaluated by pathologists to confirm the suitability of tumor cell content. Only the tumor samples that contained at least 50% tumor cells following microscopic observation using the Leica DM 2000 LED light microscope (Leica Microsystems GmbH, Wetzlar, Germany), as well as unaffected lung tissue samples without malignant cells, were used for further analysis.

RNA extraction and quality control. Total RNA was isolated from fresh-frozen tissue specimens using the mirVana miRNA isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The 100-μl resulting RNA extracts were stored at -80˚C prior to further processing. Quantity and quality of RNA assessment was performed using a UV/VIS spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, Inc.). The level of integrity required for quantitation (RNA integrity number >7) 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). A total of 500 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.) according to the manufacturer's protocol.

Quantitative polymerase chain reaction (qPCR). mRNA expression levels of *Jag1*, *Jag2*, *Dll1*, *Dll3*, *Dll4*, *Notch1* and *Hes1* were evaluated in the tumor and unaffected lung tissues using comparative qPCR. The TaqMan probes (Hs01070032_m1 *Jag1*, Hs00171432_m1 *Jag2*, Hs00194509_m1 *Dll1*, Hs01085096_m1 *Dll3*, Hs00184092_m1 *Dll4*, Hs00172878_m1 *Hes1*, Hs01062014_m1 *Notch1*) and the TaqMan Assay kit (all from Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to perform PCR. The expression of the above-mentioned genes [by the change-in-cycling-threshold ΔCq method (18,19)] were calculated and normalized to ribosomal *18SRNA* gene expression (Hs99999901_s1 *18SRNA*). The following thermocycling conditions were used: 50˚C for 2 min; 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 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.).

Statistical analysis. For statistical analysis, GraphPad Prism software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA) was used. The normality of distribution was analyzed using the Shapiro-Wilk W test. Based on the results, the following tests were used: i) Student's t-test, for normally distributed variables; ii) Mann-Whitney U test, for variables whose distributions differed from normal in at least one of the compared groups. Survival curves were created with the Kaplan-Meier method, and the log-rank test was used to determine differences between survival proportions. Cox proportional hazards model was applied to assess the prognostic strength of high or low Notch ligand expression levels. P<0.05 was considered to indicate a statistically significant difference.

Table I. Patient characteristics.

Characteristic	n	%
Gender		
Male	46	75.4
Female	15	24.6
Age at diagnosis, years		
Median (range)	61.6 (39.8-78.1)	
Mean	62.5	
Smoking history		
Smoker	54	88.5
Non-smoker	7	11.5
Histology		
Squamous cell carcinoma	25	41.0
Adenocarcinoma	28	45.9
Large cell carcinoma	8	13.1
Tumor size, T		
T1a	6	9.8
T1b	7	11.5
T2a	26	42.6
T2b	10	16.4
T3	12	19.7
Lymph node status, N		
N0	45	73.8
N1	16	26.2
Tumor stage		
IA (I)	11	18.0
IB (II)	19	31.2
IIA (III)	11	18.0
IIB (IV)	20	32.8
Follow-up period, months		
Median	49	
Range	5-86	
Status		
Alive/censored	33	54.1
Succumbed to lung cancer	27	44.3
Other cause of mortality	1	1.6
Relapse-free survival time, months		
Median	47	
Range	3-86	

Table II. Delta-like protein 3 expression in tumors.

Histology	Lack of *DLL3* expression, n (%)	Presence of *DLL3* expression, n (%)	Total, n (%)
Adenocarcinoma	14 (50)	14 (50)	28 (100)
Large cell carcinoma	5 (62.5)	3 (37.5)	8 (100)
Squamous cell carcinoma	21 (84)	4 (16)	25 (100)
Total	40 (65.6)	21 (43.4)	61 (100)

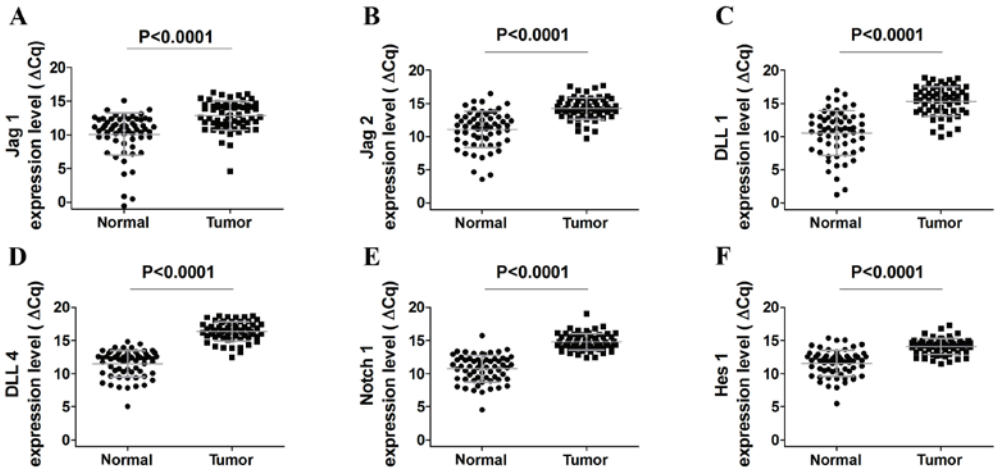

Figure 1. Gene expression level profiles of cancerous and noncancerous tissue samples. Summary of analyses of (A) *Jag1*, (B) *Jag2*, (C) *Dll1*, (D) *Dll4*, (E) *Notch1* and (F) *Hes1* expression levels in 61 noncancerous and NSCLC tissues. Results are presented as the change-in-cycling-threshold using the ΔCq method. Jag, jagged; Dll, delta-like protein; Hes1, hairy and enhancer of split-1.

Results

A total of 61 NSCLC and adjacent noncancerous lung tissue samples were analyzed by reverse transcription-qPCR. Initially, it was observed that Notch receptors and ligands were expressed in NSCLC and noncancerous lung tissue at detectable levels (Fig. 1A-E). Notably, *Jag1, Jag2, DLL1* and *DLL4* expression levels were lower in cancer when compared to the noncancerous lung tissue (all P<0.0001; Fig. 1A-F), which was indicated by increased ΔCq values. Furthermore, *DLL3* expression was consistently low and was observed only in 21/61 tumor tissues, and consequently, no significant differences in gene expression in tumor to non-tumor lung tissue were observed (data not shown). In 4/25 squamous cell carcinoma tumor samples, detectable levels of *DLL3* expression were observed. *DLL3* was expressed in 14/28 adenocarcinoma samples and in 3/8 large cell carcinoma samples (Table II).

Subsequently, the present study analyzed the expression of *Hes1*, a basic helix-loop-helix transcriptional repressor that is a downstream target of Notch signaling. Notably, *Notch 1* and *Hes1* expression was decreased in tumor samples compared to corresponding noncancerous lung tissue (both P<0.0001) and these observations were consistent with the results of the Notch ligands gene expression investigation (Fig. 1E). Furthermore, no significant differences were observed in terms of Notch receptors and ligand expression levels at various stages of the disease (Fig. 2).

Furthermore, the present study analyzed whether quantitative assessment of Notch ligand expression in NSCLC may improve patient prognostication. Having used Cox regression analysis, it was observed that increased expression of *Jag1* (above the median value observed in all NSCLC patients, 0.1478 for *Jag1*, 0.1641 for *Jag2*, 0.0590 for *Dll1*, 0.0545 for *Dll4*, 0.0791 for *Notch1* and 0.2022 for *HES1*) may serve as an indicator of poor overall survival (hazard ratio, 2.220; 95% confidence interval, 1.005-4.905; P=0.048) in NSCLC. Notably, by the use of Kaplan-Meier estimates it was demonstrated that increased *Jag1* expression in tumor tissue is associated with shortened overall survival (P=0.0422; Fig. 3A). By contrast, no statistically significant associations between survival and ligand expression for other genes were identified (Jag2, P=0.2392; DLL1, P=0.9803; DLL4, P=0.2067; Notch1, P=0.2543; HES1, P=0.3746; Fig. 3B-F).

Discussion

In the present study, the expression of Notch ligands, *Notch 1* and its target gene *Hes1*, were analyzed in NSCLC tissues. It was observed that Notch ligands, including *Jag1, Jag2, Dll1* and *Dll4*, were expressed in tumor and unaffected lung tissue samples in patients with NSCLC. By contrast, *Dll3* expression was detected in 21/61 tumor tissue samples. The results presented in the current study reveal a potentially novel role of Notch ligands in NSCLC, as it was demonstrated that the

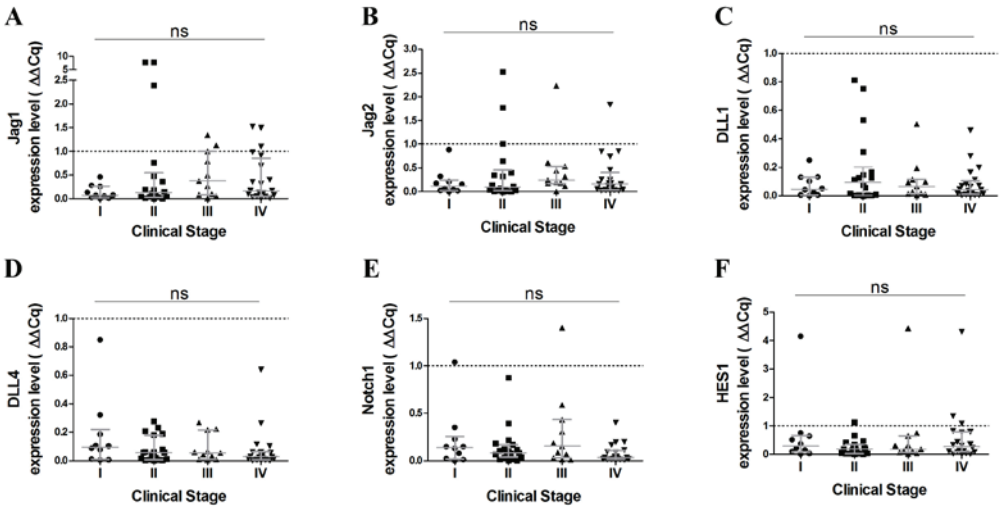

Figure 2. Associations between Notch signaling genes expression (assessed by ΔΔCq) and clinical stages of NSCLC patients. Association of (A) *Jag1*, (B) *Jag2*, (C) *Dll1*, (D) *Dll4*, (E) *Notch1* or (F) *Hes1* expression level and the clinical stages of NSCLC. Jag, jagged; Dll, delta-like protein; Hes1, hairy and enhancer of split-1; ns, not significant.

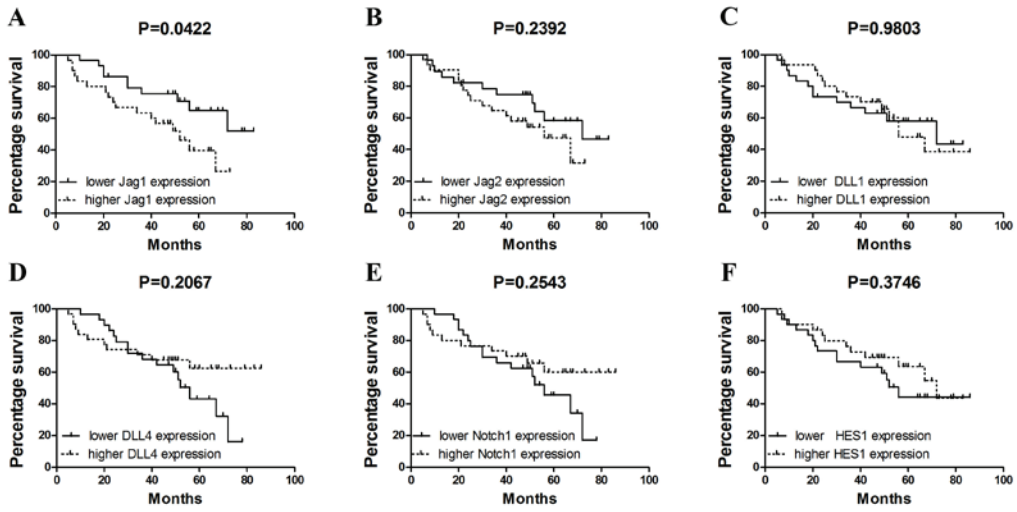

Figure 3. Kaplan-Meier estimates for expression levels of (A) *Jag1*, (B) *Jag2*, (C) *Dll1*, (D) *Dll4*, (E) *Notch1* and (F) *Hes1* divided into two groups above (lower, solid line) and below (higher, dotted line) the median value. Jag, jagged; Dll, delta-like protein; Hes1, hairy and enhancer of split-1.

Notch signaling pathway is significantly deregulated in NSCLC patients. The levels of Notch ligands were consistently lower in tumor tissues, when compared to adjacent noncancerous lung tissues from the same patient. Thus, the present study aimed to evaluate the potential role of the analyses of tumor-associated alterations of Notch signaling in prognostication of NSCLC patients. The identification of novel prognostic markers capable of predicting overall survival and clinical consequences of applied therapies is required in NSCLC. The present study demonstrated that increased expression of *Jag1* may be an indicator of poor overall survival. This finding is notable as tumor tissues were characterized by reduced Notch signaling levels compared to noncancerous tissues, but increased expression of *Jag1* indicated a less favorable clinical outcome. Thus, the results of the present study add another piece of evidence to the complex role of Notch signaling in the pathogenesis of NSCLC.

In certain ways, the present findings do not support the results of a recent meta-analysis performed by Yuan *et al* (20) who demonstrated that increased expression of *Notch 1* was more frequently accompanied by lymph node metastasis and more advanced TNM stage. Consistently, Yuan *et al* (20) observed that patients with *Notch 1* or *Notch 3* overexpression presented with significantly poorer overall survival. Similarly, in another recent study, *Notch 1* overexpression was observed to increase the metastatic potential of NSCLC cells (21). To a certain extent, these findings were contrasted by Nguyen *et al* (22) who discovered that the expression of Notch 1 receptors in NSCLC tissues was negatively associated with stage and nodal status, but not tumor size. Notably, the expression of activated form of *Notch 1*, N1-ICD (intracellular domain) was low and neither significantly associated with stage nor nodal status (22). To date, it has been postulated that the effects of Notch signaling

contributing to maintaining a balance between cell proliferation and apoptosis may be either oncogenic or tumor-suppressive depending on the cancer type (7). In the present study, it may be hypothesized that these effects differ even within a single cancer type. Similarly, Notch-associated signals have dual negative and positive (oncogenic and tumor-suppressive) roles in the course of hematological malignancies (23). In the present study, reduced expression levels of Notch ligands and receptors in tumor tissues were consistently observed compared to control samples. By contrast, increased expression levels of *Jag1* were observed to be positively associated with a less favorable clinical outcome.

The results of the present study warrant additional studies on whether downregulation of Notch ligands and receptors is induced by tumors in order to suppress endogenous anti-tumor mechanisms, or if it represented a mechanism of self-defense against the developing tumor. The idea of downregulation of Notch signaling is novel for lung cancer but it is not unexpected in the light of studies performed on other types of cancer, including endometrial cancer. Jonusiene *et al* (24) investigated the expression of Notch receptors (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*), ligands (*Jag1*, *Jag2* and *Dll1*) and target gene *Hes1* in endometrial cancer and adjacent non-tumor endometrial tissue from endometrial cancer patients. The mRNA levels of Notch receptors and ligands were reduced in endometrial cancer compared with adjacent non-tumor tissue (24). Furthermore, in contrast to the results of the present study, the expression of *Notch1*, *Notch4* and *Dll1* in IB stage adenocarcinoma was significantly reduced compared with the expression of these molecules at the IA stage (16). Considered along with the results of the present study, these findings suggest that Notch-mediated signaling may support tumor-suppressing mechanisms in certain types of cancer, including lung or endometrial cancer.

In conclusion, the present study reported that Notch 1 and Notch ligands are downregulated in tumors when compared to noncancerous lung tissue in NSCLC patients. Furthermore, the present study demonstrated that quantitative measurement of *Jag1* expression may improve prognostication of NSCLC patient survival. In contrast to the work of previous authors, the present study hypothesizes that the role of Notch signaling in the pathogenesis of NSCLC cannot be simply linked to either upregulation or downregulation of its ligands and receptors in tumor tissue. The identification of alternative factors influencing Notch-related signaling pathways in the settings of NSCLC remains warranted.

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