Correlation and Coexpression of HIFs and NOTCH Markers in NSCLC

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Abstract. *Background: NOTCH and hypoxia pathways are both known to be highly involved in cancer. Because of the close interplay between both of these pathways, we investigated correlation and co-expression of molecules in these pathways. Materials and Methods: In 335 unselected stage I-IIIA NSCLC patients, protein expressions of hypoxia inducible factor 1α (HIF1α), hypoxia inducible factor 2α (HIF2α), glucose transporter 1 (GLUT1), lactate dehydrogenase 5 (LDH5), carbonic anhydrase IX (CAIX), delta like 4 (DLL4), JAGGED1, NOTCH1 and NOTCH4, evaluated by immunohistochemistry, were correlated and co-expressions tested in tumor and stromal cells. Results: HIF2α and LDH5 correlated moderately with DLL4, JAGGED1 and NOTCH4 in both tumor and stromal compartments (Spearman's r=0.16-0.33). The coexpression of HIF1α and NOTCH1 in tumor was significantly indicative of poor prognosis in univariate analysis. Hypoxia and NOTCH ligands and receptors were moderately correlated. Conclusion: The lack of appealing coexpression findings for HIF1α and NOTCH1 may be due to the way HIF1α directly influences NOTCH signalling without depending on an elevated NOTCH expression.*

Identification of new potential targets for therapeutic intervention is vital in the quest for an effective future treatment of Non-small Cell Lung Cancer (NSCLC). Successful targeting in NSCLC will require a considerable refinement of our understanding of pathways and crosstalk in both normal and malignant cells.

The master regulator of hypoxic adaptation, hypoxia inducible factor 1α (HIF1α) and molecules involved in proliferation and differentiation (NOTCH) have all been proposed as targets of molecular therapy (1, 2), and both pathways are known to be deregulated in cancer (2, 3). In addition to its importance in orchestrating angiogenesis, erythropoiesis and altering metabolism, HIF1α is recruited to a NOTCH-responsive promoter leading to an elevated expression of NOTCH downstream genes, blocking differentiation and increasing proliferation (4).

We have earlier reported on the prognostic impacts of the expression of hypoxia-related proteins (5) and NOTCH ligands and receptors (6). Given the close interplay between these pathways, we investigated the combined protein expression profiles of these markers in a large cohort of NSCLC patients in order to assess clinical significant correlations or co-expressions.

Patients and Methods

Primary tumor tissues from 335 unselected patients surgically resected for pathological stage I to IIIA NSCLC at the University Hospital of Northern Norway and Nordland Central Hospital from 1990 to 2004 were histologically reviewed by two pathologists, with selection of the most representative areas of neoplastic cells (tumor) and tumor-related stroma (stroma). Two core biopsies from tumor and two from stroma were collected from each surgical specimen. All core biopsies were inserted in recipient tissue microarray (TMA) blocks. By light microscopy, representative and viable tissue sections were scored semiquantitatively for cytoplasmic staining of immunohistochemistry (IHC). The dominant staining intensity in both tumor cells and stromal cells was scored as: 0=negative; 1=weak; 2=intermediate; 3=strong. The cell density of the stroma was scored as: 1=low density; 2=intermediate density; 3=high density. Cut-off values were set individually for each marker. Information regarding IHC procedures, antibodies and IHC scoring has been described thoroughly previously (5, 6).

The SPSS version 15.0.1.1 from SPSS Inc, (Chicago, IL, USA) was used for the statistical analyses. The Chi-square test was used to examine associations between molecular marker expression, and $p<0.05$ was considered statistically significant. The Spearman's rank correlation coefficient was used as the measure of statistical

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Table I. *Correlation between expression of hypoxic markers and NOTCH markers in NSCLC.*

Molecular marker		DLL4		JAGGED1		NOTCH1*	NOTCH4	
	Compartment	Tumor	Stroma	Tumor	Stroma	Tumor	Tumor	Stroma
HIF1α	Tumor	p=0.75		p=0.017 r=0.13		p=0.94	p=0.39	
	Stroma		p=0.09		p<0.001 r=0.184			p=0.37
HIF2α	Tumor	p=0.0058 r=0.33		p<0.001 r=0.29		p=0.78	p<0.001 r=0.31	
	Stroma		p<0.001 r=0.27		p<0.001 r=0.26			p<0.001 r=0.30
LDH5	Tumor	p=0.0058 r=0.16		p<0.001 r=0.29		p=0.777	p<0.001 r=0.31	
	Stroma		p<0.001 r=0.23		p<0.001 r=0.26			p<0.001 r=0.25
CAIX‡	Tumor	p=0.94		p=0.21		p=0.076	p=0.22	
GLUT1†	Tumor	p=0.075		p=0.94		p=0.26	p=0.98	

*NOTCH1 was not scored in stroma due to lack of variation; †Only 3 stromal cores had high expression for GLUT1; ‡There was no expression of CAIX in stroma; r is the Spearman correlation and is only stated in cases of significance.

dependence of the variables. The Kaplan-Meier method was used for univariate testing and statistical significance between survival curves was assessed by the log-rank test. To assess the independent impact of coexpression on survival, significant clinicopathological, marker and coexpression variables from the univariate analysis were entered into the multivariate Cox regression analysis. The significance level used was p<0.05.

Results

Patient characteristics. This report includes follow-up data as of November 30, 2008. The median follow-up of survivors was 86 (range 48-216) months. The median age was 67 (range 28-85) years and the majority of patients were male (75%). The NSCLC tumors comprised 191 squamous cell carcinomas, 113 adenocarcinomas and 31 large cell carcinomas. Prognostic clinicopathological variables were WHO performance status (p=0.013), differentiation (p<0.001), surgical procedure (p=0.004), pathological stage (p<0.001), tumor stage (p=0.002), nodal stage (p<0.001) and vascular infiltration (p<0.001). Detailed patient and expression data can be found in previous publications (5;6).

Correlation between molecular markers expression. Details on the correlation between different marker expressions are presented in Table I. Both HIF2α and LDH5 correlated significantly with DLL4, JAGGED1 and NOTCH4 in both the tumor and stromal compartments. HIF1α was weakly, but significantly correlated to JAGGED 1 in both tumor and stromal cells.

Coexpression of markers. All coexpression combinations of hypoxic and NOTCH markers in tumor cells were assessed by univariate and multivariate analyses. None of these coexpressions gave any additional information when related to the expression of either marker. The univariate analyses of survival according to HIF1α, NOTCH1 and coexpression of these are presented in Figure 1. Multivariate analyses for the coexpression variables with significant clinicopathological variables are shown in Table II. The co-expression subgroup of ↑NOTCH1/↓HIF1α was an independent and significant poor prognosticator compared with ↓NOTCH1/↓HIF1α (Hazard Ration (HR) 2.97, 95% Confidence interval (CI) 1.63-5.42; p<0.001). When we entered only the variable HIF1α along with the clinicopathological variables we found high expression of HIF1α to be an independent poor prognosticator for disease-specific survival (HR 2.08, 95% CI 1.20-3.59; P=0.009). When the same analysis was carried out for NOTCH1 alone, the result was non-significant (HR 1.28, 95% CI 0.88-1.87; p=0.20).

Discussion

Evaluating hypoxia markers and NOTCH ligands and receptors in our high-throughput NSCLC TMAs, we found moderate and significant correlations between HIF2α and NOTCH ligands DLL4 and JAGGED1 and NOTCH receptor NOTCH4 in both tumor and stroma. LDH5 was correlated moderately to the same ligands and receptors as HIF2α, but was in addition correlated to NOTCH1 in tumor. Surprisingly HIF1α was only significantly correlated to the NOTCH ligand

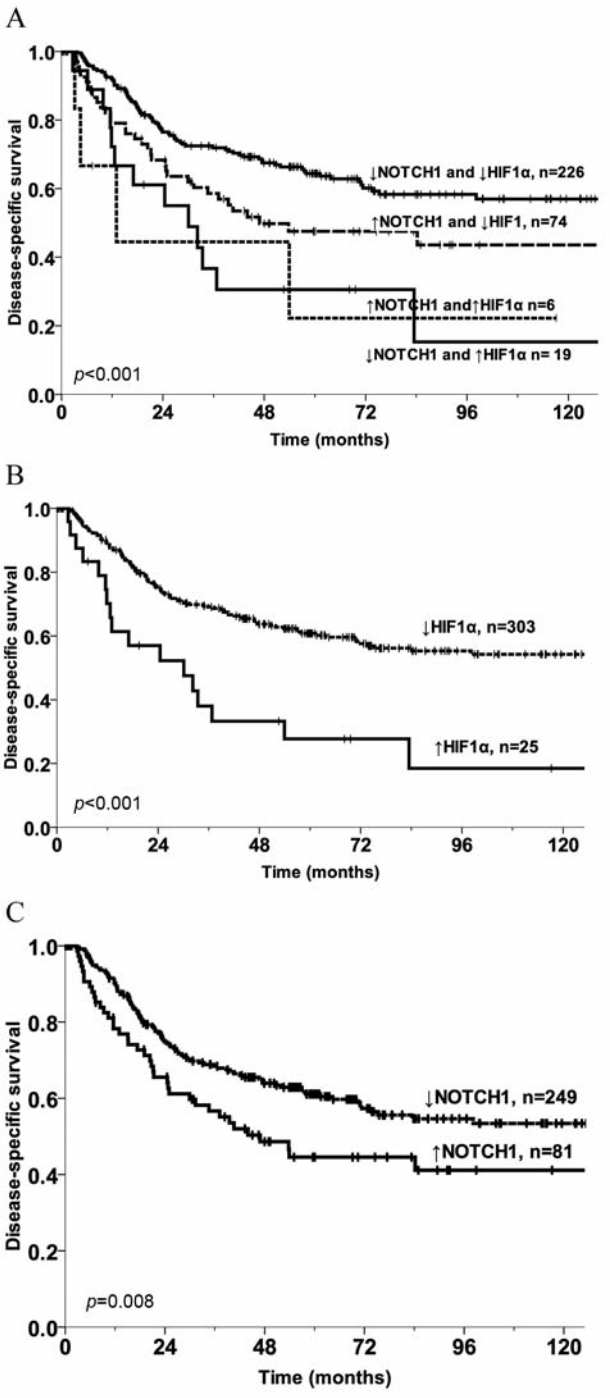

Figure 1. *Disease-specific survival curves according to: A: coexpression of tumor cell HIF1α and NOTCH1; B; tumor cell HIF1α expression; C: tumor cell NOTCH1 expression.*

JAGGED1 in both tumor and stroma. Using coexpression data of both markers, we found no rationale for clinical synergism between elevated protein expression levels of hypoxia and NOTCH markers.

Table II. *Multivariate analysis of disease-specific survival including all significant clinicopathological variables and tumor cell coexpression of HIF1α and NOTCH1.*

Variable	Hazard ratio	*p*
Coexpression		0.003*
↓NOTCH1/↓HIF1α	1	
↑NOTCH1/↓HIF1α	2.97 (1.63-5.42)	<0.001
↓NOTCH1/↑HIF1α	NS	NS
↑NOTCH1/↑HIF1α	NS	NS
Performance status		0.002*
0	1	
1	1.90 (1.30-2.77)	0.001
2	2.23 (1.003-4.94)	0.049
Differentiation		0.010*
Well	1	
Moderate	NS	
Poor	NS	
Tumor stage		0.011*
1	1	
2	1.85 (1.16-2.94)	0.01
3	2.50 (1.28-4.89)	0.008
Nodal stage		<0.001*
0	1	
1	1.97 (1.32-2.94)	0.001
2	2.73 (1.54-4.81)	0.001
Vascular infiltration		0.001
No	1	
Yes	2.26 (1.42-3.60)	

*Overall significance as a prognostic factor; NS, not significant in analysis. Numbers in brackets are 95% confidence intervals.

In NSCLC, no other studies have evaluated correlation and coexpression of these markers. Jubb *et al*. investigated the expression of hypoxia markers against DLL4 in a colon cancer study, but they found that DLL4 did not correlate to HIFs (7).

In breast cancer cell lines *in vitro*, Chen and co-workers found that hypoxia, probably through accumulation of HIF1α and HIF2α, increased the expression of NOTCH receptors and ligands as well as NOTCH target genes (8). Stabilization of HIF1α has also been revealed to increase mRNA and protein levels of NOTCH1 in melanoma cell lines (9). In lung cancer cell lines, hypoxic conditions dramatically elevated NOTCH signaling (especially of NOTCH1) compared to cell lines cultured under normoxia (10).

How can our results of only moderate correlation between HIF2α and NOTCH ligands and receptors be interpreted in the context of these *in vitro* data? Firstly, IHC is a snapshot of the tissue's molecular expression and involved molecules may not be expressed at high levels simultaneously. HIFs are also known to be rapidly degraded (11) and differentially expressed during prolonged hypoxia (12). In the tissues, other transcription factors may also be involved, blurring the clear-cut image we have of the pathways involving hypoxia and NOTCH markers.

Regarding our coexpression findings, Gustafsson and colleagues found that HIF1α interferes with the NOTCH pathway in a hypoxic non-canonical manner by interacting with intracellular NOTCH directly, acting in synergy with the NOTCH intracellular domain with subsequent transcription of NOTCH targets (4). Hypoxia activates NOTCH-responsive promoters and increases expression of NOTCH downstream genes. Chen and co-workers demonstrated that hypoxia potentiated the strength of NOTCH signaling even under levels of low intracellular NOTCH1 (8). The NOTCH intracellular domain interacts with HIF-1α, a global regulator of oxygen homeostasis, and HIF-1α is recruited to NOTCH-responsive promoters upon NOTCH activation under hypoxic conditions. Accordingly, there may be synergism without the need for elevated protein levels of NOTCH markers. Actual NOTCH1 signaling may be largely unrelated to the immunohistochemically detected NOTCH1 expression levels due to the strong drive HIF1α has on NOTCH signaling. Our results add clinical relevance for these experimental finding as patients with elevated HIF1α do not have their prognosis aggravated by additionally elevated NOTCH1 levels. Thus, the only patient population with functionally low NOTCH1 signaling may be the large group of patients (n=226, 65%) with the low NOTCH1/low HIF1α combination.

Although the prognostic effect of high HIF1α/high NOTCH1 coexpression does not add prognostic information compared to that of high HIF1α expression alone, this is a small group of patients and an elevated level of NOTCH1 has a prognostic effect in the large group of patients with a low expression of HIF1α. From a therapeutic perspective, NOTCH inhibition has proved to be a plausible therapeutic strategy, especially under hypoxic conditions. However, IHC detection of HIF1α has not proven to be a reliable marker of hypoxia. By direct measurements during surgery, a larger proportion of tumors than identified by our IHC study were found to be hypoxic (13). Thereby HIF1α expression alone may not be an adequate means to identify patients with hypoxic tumors prone to have NOTCH signaling inhibition.

In summary, there are correlations between HIFs and NOTCH ligands and receptors, which were more prominent for HIF2α than for HIF1α. Coexpression of HIF1α and NOTCH1 showed that patients with any combination of high HIF1α expression had a worse prognosis, and that NOTCH1 appeared to have a role only in the subgroup with low HIF1α expression. This could be because of the way HIF1α interacts directly in the NOTCH signaling pathway.

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