

The Role of the Homeobox Genes BFT and CDX2 in the Pathogenesis of Non-small Cell Lung Cancer

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Abstract. *Background:* The role of the homeobox genes Backfoot (BFT) and caudal-related Homeobox 2 (CDX2) in the pathogenesis of non-small cell lung cancer (NSCLC) is unclear. The goal of this study was to investigate the mRNA expression of BFT and CDX2 in NSCLC and to determine the association with the pathogenesis and the potential as a biomarker of this disease. *Materials and Methods:* The mRNA expression of BFT and CDX2 was analyzed by quantitative real-time RT-PCR in the tumor and matching normal tissue from 23 patients with NSCLC. *Results:* The mRNA expression was detectable with the following frequencies in the tumor (*t*) and normal (*n*) tissues: BFT=100% (*n*), 100% (*t*); CDX2=100% (*n*), 100% (*t*). The median CDX2 mRNA expression was 0.85 (range: 0.01-15.47) in the tumor tissue and 0.045 (range: 0-1.36) in the matching normal lung tissue (*p*=0.001). The median BFT mRNA expression was 0.0034 (range: 0-0.35) in the tumor tissue and 0.0001 (range: 0-0.10) in the matching normal lung tissue (*p*=n.s.). There were no associations between the mRNA expression levels of BFT and CDX2 and clinicopathological variables. *Conclusion:* The mRNA expression of the homeobox genes is detectable at a high frequency in the tumor and normal tissue of patients with non-small cell lung cancer. Up-regulation of CDX2 mRNA expression appears to be associated with the pathogenesis of this malignant disease. The quantification of CDX2 and BFT mRNA expression in lung tissue is a potential biomarker for the identification of patients at risk of the development of NSCLC.

Lung cancer is the leading cause of cancer-related death in the United States, accounting for more deaths than from prostate, breast and colorectal cancer combined. In the United

States, the estimated number of deaths due to lung cancer alone was estimated to exceed 100,000 in 2008 (1). Radical surgery offers the only chance of a cure in patients with non-small cell lung cancer (NSCLC), but despite improvements in the detection and treatment of this disease, the 5-year survival rate remains less than 15% (2). To further improve the outcome of patients with NSCLC, it is evident that additional techniques, markers and medicines are needed to diagnose, stratify and treat patients with lung cancer.

The caudal-related homeobox gene 2 (CDX2) and backfoot, or pituitary homeobox gene 1 (BFT or PITX1) are members of the homeobox gene family, nuclear transcription factors that have an essential role as regulatory proteins for proliferation and differentiation of intestinal epithelial cells in fetal and adult tissues (3). CDX2 is expressed specifically in colonic and small intestinal mucosa and has been implicated in disorders involving abnormal intestinal differentiation and neoplasia (4, 5). The use of CDX2 as a potential molecular marker has been proposed for several malignancies, including gastric, colonic and esophageal cancer (6-8). Furthermore, CDX2 expression has been investigated as a marker to reliably identify the colorectal origin of lung metastasis (9, 10). However, the role of CDX2 mRNA expression in the pathogenesis of NSCLC remains unsolved.

BFT encodes a member of the bicoid subgroup of paired homeobox-containing transcription factors (11, 12) and plays a crucial role in the development of specific cell types in the pituitary gland (13-16). BFT is expressed at an early stage in the developing oral squamous epithelium and in the duodenum (16). Furthermore, expression of BFT is suggested to play a crucial role in lung development (17). Recently, one study has reported that BFT expression might be linked to lung cancer development and progression (18).

The aim of this study was to analyze the mRNA expression of CDX2 and BFT in tumor tissues and adjacent normal lung tissues obtained from patients with NSCLC and to determine the role of these homeobox genes in the pathogenesis of this disease.

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Key Words: BFT, CDX2, homeobox genes, gene expression, NSCLC.

Materials and Methods

Sample collection. The study specimens were paired tumor and normal lung tissues from 23 patients with NSCLC. There were 17 (74%) men and 6 (26%) women, with a median age of 63.3 years (range 34-82 years). Nine (39%) of the patients had squamous cell carcinomas, 8 (35%) had adenocarcinomas and 6 (26%) had large cell carcinomas. The primary tumors were graded histopathologically as well-differentiated (G1, one patient), moderately-differentiated (G2, 3 patients) and poorly-differentiated (G3, 19 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification (19): 14 (61%) had stage I tumors, 5 (22%) had stage II tumors and 4 (17%) had stage IIIa tumors. All the tumors were completely resected (R0 resection). The patients with histopathological stage IIIa tumors received postoperative radiotherapy. Informed consent was obtained from all the patients.

The tissue for mRNA expression analysis was obtained immediately after lung resection before starting mediastinal lymphadenectomy and was immediately frozen in liquid nitrogen and stored at -80°C. Tissue was analyzed from the tumor and from the uninvolved lung tissue taken at the greatest distance from the tumor. Six µm frozen sections were taken from the blocks of tumor tissue and starting with the first section every 5th was routinely stained with hematoxylin-eosin (H&E) and histopathologically evaluated. The sections were pooled for the analysis of the histologically normal lung tissues and areas of estimated 75% malignant cells were used for the matching tumor analysis.

Isolation of mRNA. Total RNA was isolated by a single-step guanidinium isothiocyanate method using a QuickPrepMicro mRNA Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. After RNA isolation, cDNA was prepared from each sample as described previously (20, 21).

Real-time RT-PCR quantification. Quantitation of *CDX2*, *BFT* and an internal reference gene β -actin was conducted using a fluorescence based real-time detection method (ABI PRISM 7700 Sequence Detection System [Taqman], Applied Biosystems, Foster City, CA, USA) as previously described (22, 23). The PCR reaction mixture consisted of 600 nmol/L of each primer, 200 nmol/L probe, 2.5 U AmpliTaq Gold Polymerase, 200 µmol/L each of dATP, dCTP and GTP, 400 µmol/L dUTP, 5.5 mmol/L MgCl₂ and 1 x Taqman Buffer A containing a reference dye, to a final volume of 25 L (all reagents, Applied Biosystems). Cycling conditions were 50°C for 10 seconds and 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primers and probe sequences used were as follows: *CDX2* forward primer 5'-ACC AGG ACG AAA GAC AAA TAT CGA-3', reverse primer 5'-TGT AGC GAC TGT AGT GAA ACT CCT TCT-3', probe 6FAM-5'-TGT ACA CGG ACC ACC AGC GGC TG-3'-TAMRA; *BFT* forward primer, 5'-ACC AAG AGC TTC ACC TTC TTCAA-3', reverse primer 5'-CTG GGT GCT GAG AAC ATG GA-3', probe 6FAM 5'-TCC ATG AGC CCG CTG TCG TCG-3'TAMRA and β -actin forward primer 5'-TGA GCG CGG CTA CAG CTT-3', reverse primer 5'-TCC TTA ATG TCA CGC ACG ATT T-3', probe 6FAM5'-ACC ACC ACG GCC GAG CGG-3'TAMRA. For each sample, parallel Taqman PCR reactions were performed for each gene of interest and the β -actin reference gene to normalize for input cDNA. The ratio between the values obtained provided the relative gene expression level for the gene locus investigated.

Table I. Association between *CDX2* and *BFT* expression in tumor tissue and clinicopathological variables.

Parameter	n	Median CDX2 expression (range)	P	Median BFT expression (range)	P
Gender			n.s.		n.s.
Male	17	0.70 (0.04-5.2)		0.0035 (0.0-0.3)	
Female	6	0.96 (0.01-15.4)		0.0021 (0.0-0.3)	
Smoker			n.s.		n.s.
Yes	19	0.85 (0.01-15.4)		0.0034 (0.0-0.3)	
No	4	0.80 (0.1-1.1)		0.0025 (0.0-0.1)	
T status			n.s.		n.s.
pT1	6	0.91 (0.3-5.2)		0.0034 (0.0-0.3)	
pT2	16	0.88 (0.01-15.4)		0.0033 (0.0-0.3)	
pT3	1	0.70 (0.1-0.7)		0.0001 (0.0-0.1)	
N Status			n.s.		n.s.
pN0	15	0.68 (0.01-15.4)		0.0034 (0.0-0.3)	
pN1	4	1.00 (0.1-1.8)		0.0019 (0.0-0.1)	
pN3	4	0.96 (0.7-3.2)		0.0130 (0.0-0.3)	
Histology			n.s.		n.s.
Squamous	9	0.68 (0.04-15.4)		0.0034 (0.0-0.3)	
Adeno	8	0.98 (0.3-1.1)		0.0025 (0.0-0.3)	
Large cell	6	0.52 (0.01-1.8)		0.0038 (0.0-0.2)	
Tumor stage			n.s.		n.s.
I	14	0.80 (0.04-15.4)		0.0034 (0.0-0.3)	
II	5	0.85 (0.01-1.8)		0.0014 (0.0-0.01)	
IIIa	4	0.96 (0.70-3.1)		0.0130 (0.0-0.3)	
Grading			n.s.		n.s.
Well	1	0.39		0.0006	
Moderate	3	0.68 (0.01-3.1)		0.0208 (0.0-0.3)	
Poor	19	0.91 (0.01-15.4)		0.0034 (0.0-0.3)	

n.s., Not significant; n, number of patients.

Statistical analysis. Associations between two related variables were tested by using the Wilcoxon signed rank test. The Mann-Whitney test and Kruskal-Wallis test were used to evaluate the associations of *CDX2* and *BFT* expression (as continuous variables) with the patient's clinicopathological variables. Associations between continuous mRNA expression levels were tested for correlation by Spearman's test. The level of significance was set to *p*<0.05. All the *p*-values reported were based on two-sided tests.

Results

The mRNA expression was detectable with the following frequencies in tumor (t) and normal (n) tissues: *BFT*=100% (n), 100% (t); *CDX2*=100% (n), 100% (t) and β -actin=100% (t), 100% (n).

The median *CDX2* mRNA expression in relation to the internal reference gene β -actin was 0.85 (range: 0.01-15.47) in the tumor tissues and 0.045 (range: 0-1.36) in the matching normal lung tissue (*p*=0.001; Wilcoxon test; Figure 1). There were no associations between *CDX2* mRNA expression levels and the clinicopathological variables (Table I).

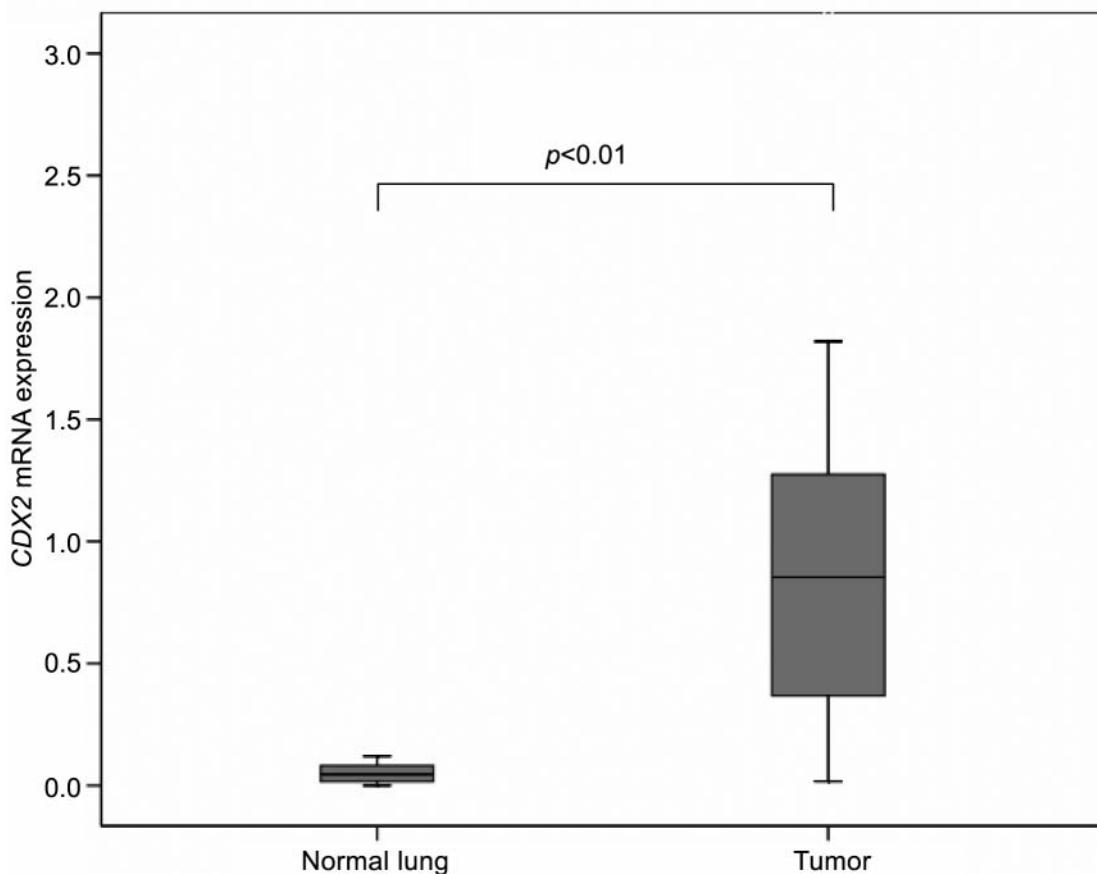


Figure 1. Box and whisker plots of relative CDX2 expression levels in tumor and normal lung tissues. The boxes show the 25th and 75th percentile (interquartile) ranges. Median values are shown as a horizontal black bar within each box. The whiskers show levels outside the 25th and 75th percentiles, but exclude far outlying values.

The median *BFT* mRNA expression in relation to β -actin was 0.0034 (range: 0-0.35) in the tumor tissue and 0.0001 (range: 0-0.10) in the matching normal lung tissue ($p=n.s.$; Wilcoxon test; Figure 2). There were no associations between the relative *BFT* mRNA expression levels and the clinicopathological variables (Table I).

To further investigate the role of these genes in the pathogenesis of lung cancer, the relationship between their expressions was determined. A scatter plot of *CDX2* against *BFT* expressions (Figure 3) showed no correlation between the expressions of these genes in the tumor tissues ($r^2=0.158$; $p=0.471$; Spearman's test).

Discussion

The altered expression of the homeobox genes *CDX2* and *BFT* was a frequent event in NSCLC in this study. Recent reports have shown that *CDX2* expression is not restricted to metastasis of colorectal origin, but is readily detectable in primary lung adenocarcinomas by real-time PCR and by

immunohistochemistry (24, 25). The present study was in agreement in terms of *CDX2* expression in primary adenocarcinomas of the lung, but in addition the expression of this homeobox gene was found to be positive in 100% of the primary squamous cell carcinoma and large cell carcinoma of the lung. There are several possible explanations for these findings. Firstly, these variant results are most likely attributable to the reported 10-fold higher sensitivity of the quantitative real-time RT-PCR used in our study compared with conventional PCR techniques (26). Secondly, *CDX2* mRNA expression has been detected in tissues other than those of gastrointestinal origin, for example renal cancer, squamous cell cancer of the lung, hepatocellular and, ovarian carcinoma and cancer of the esophagus (7, 24). These data suggest that *CDX2* has a wide range of expression in human carcinomas and is not restricted to certain histologies.

Furthermore, *CDX2* mRNA expression was detected in 100% of the normal lung tissues of the patients with NSCLC, although at a significantly lower level compared to the

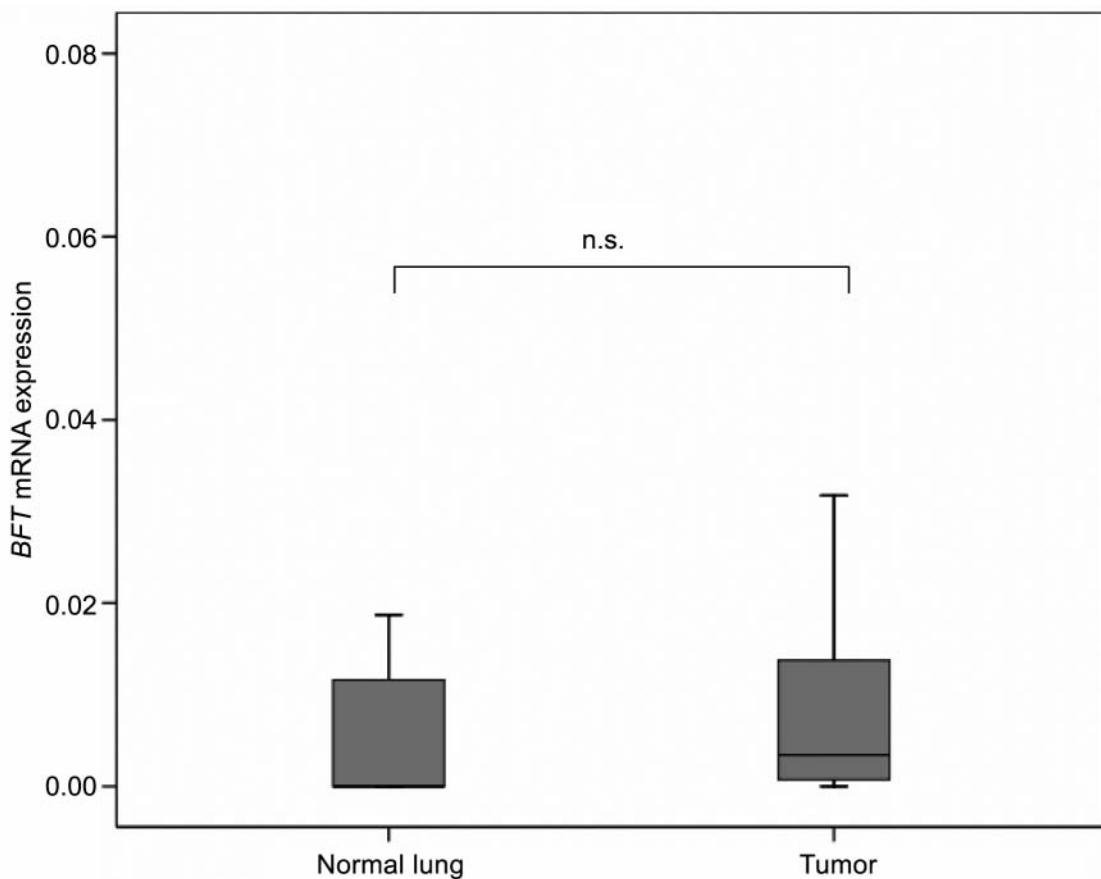


Figure 2. Box and whisker plots of relative *BFT* expression levels in tumor and normal lung tissues. The boxes show the 25th and 75th percentile (interquartile) ranges. Median values are shown as a horizontal black bar within each box. The whiskers show levels outside the 25th and 75th percentiles, but exclude far outlying values.

matching tumor tissues. *CDX2* expression has previously been observed in normal human tissues, for example normal rectal epithelium or normal squamous epithelium of the esophagus (7, 24). These results and the markedly altered expression of both genes in the present study suggested that they may have a mechanistic role in the development and progression of human neoplasia, but objective evidence for this role was not examined in the present study. Other homeobox genes have been implicated in neoplastic development, and there is increasing support for the ‘oncofetal’ theory of the relationship between tumorigenesis and embryogenesis (27-29).

In contrast to *CDX2*, the association of cancer development and *BFT* expression has been investigated less frequently. Our group was the first to report a correlation between decreased *BFT* mRNA expression and the pathogenesis of Barrett’s associated cancer of the esophagus (7). Recently, Chen and co-workers were the first to demonstrate altered *BFT* expression in primary lung cancer, suggesting that *BFT* might be linked to lung cancer development and progression (18).

Furthermore, *BFT* was found to be decreased in other malignancies, for example prostate and bladder cancer, suggesting *BFT* as a tumor suppressor gene in human carcinomas (30). Although markedly altered in the tumor tissues compared to the normal lung tissue in the patients with NSCLC, the difference in gene expression was statistically not significant in the present study.

In conclusion, the data confirm other reports of markedly altered expression of the homeobox genes *CDX2* and *BFT* compared with normal tissue in human carcinomas. The hypothesis that *CDX2* and *BFT* play a crucial role in the pathogenesis of NSCLC is strongly suggested. Future studies are warranted to further elucidate the role of these genes in human carcinogenesis.

Acknowledgements

This study was supported in part by a grant from the Köln Fortune Stiftung, Cologne, Germany.

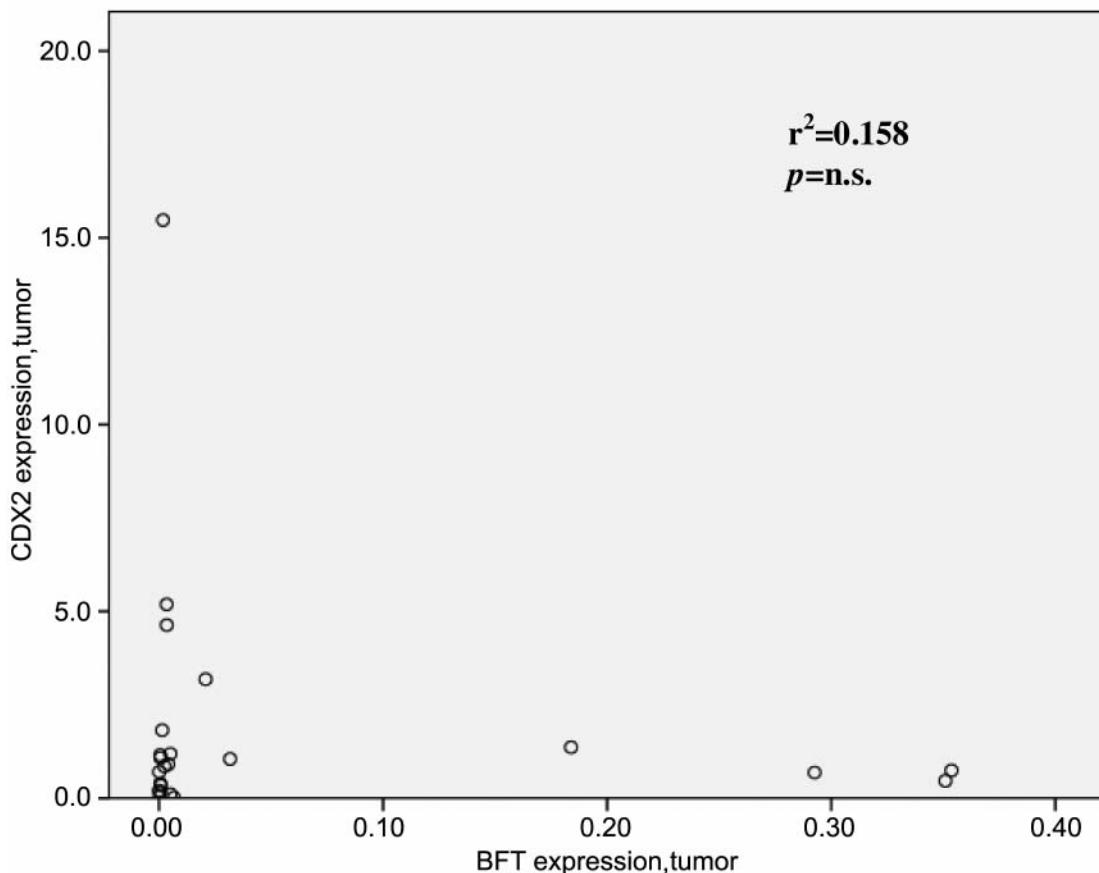


Figure 3. Scatter plot of CDX2 expression against BFT mRNA expression in tumor tissues of patients with NSCLC.

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*Received November 24, 2008**Revised January 23, 2009**Accepted February 13, 2009*