JHC

Prognostic Significance of Wnt1, Wnt2, E-Cadherin, and β-catenin Expression in Operable Non-small Cell Lung Cancer

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Summary

The role of Wnt family proteins, E-cadherin, and β-catenin in non-small cell lung cancer (NSCLC) is unclear. In this study, we assessed the expression of these proteins as well as their reciprocal interaction and clinical relevance in NSCLC. Immunohistochemical expression of Wnt1, Wnt2, E-cadherin, and β-catenin was assessed in 208 patients with NSCLC who underwent curative pulmonary resection. Expression of Wnt1, Wnt2, and E-cadherin was found in 49.5%, 22.3%, and 37.4% of the patients, respectively, whereas expression of membranous and cytoplasmic β-catenin was found in 23.7% and 34.8% of the patients, respectively. The expression of Wnt1 and E-cadherin was lower in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma, and the expression of both Wnt proteins, E-cadherin, and membranous β-catenin was lower in poorly differentiated compared with well-differentiated tumors. None of the analyzed proteins was associated with relapse-free or overall survival. Expression of Wnt1, Wnt2, E-cadherin, and β-catenin is a common occurrence in NSCLC and is related to tumor histology and grade. However, these proteins have no prognostic role in operable NSCLC. (J Histochem Cytochem 69: 711–722, 2021)

Introduction

Non-small cell lung cancer (NSCLC) is the most common malignancy worldwide. Its treatment outcomes remain unsatisfactory, with an overall 5-year survival probability of 10–14% in the European Union.[1] Most patients are diagnosed at advanced stages. Classical cytotoxic treatment of advanced disease is highly ineffective, with a response rate of approximately 30% and median progression-free survival in the range of 3–6 months.[2] Therefore, better understanding of lung cancer biology may improve this grim picture. Indeed, the discovery of several driving molecular aberrations in lung cancer has paved the way for the development of specific targeted therapies.

One of the signaling pathways that has drawn attention is that driven by wingless-type (Wnt) family genes. The canonical signaling pathway dependent on Wnt glycoproteins and β-catenin was discovered in the 1980s.[3,4] The activity of this pathway is determined by the level of β-catenin accumulated in the cytoplasm.[5,6] Physiologically, the cytoplasmic level of β-catenin is kept low by the continuous proteosomal degradation.[7] Signal transduction through this canonical pathway starts with the binding of Wnt1 and Wnt2 ligands with Fzd and low-density lipoprotein receptor-related protein (LRP) receptors, leading to the inhibition of β-catenin phosphorylation.[8] Unphosphorylated β-catenin then accumulates in the cytoplasm and is subsequently translocated to the nucleus, where it creates complexes

with transcriptional factors (Tcf/Lef) and promotes gene expression (*cyclin D1*, *c-myc*, *MMP7*, *VEGF-A*, and *survivin*).[9–12] At the cellular level, β-catenin also interacts with E-cadherin and forms the adherens junctions that are responsible for intercellular adhesion.[13–15] Dysfunction of this interaction promotes stromal invasion and metastatic potential. Activation of the Wnt signaling pathway in epithelial stem cells may lead to the induction of neoplasms, inhibition of cancer cell apoptosis, increased tumor aggressiveness, and tumor resistance to chemotherapy and radiation.[16–18] This study is the first comprehensive immunohistochemical (IHC) assessment of two main Wnt family proteins, E-cadherin, and β-catenin, as well as their reciprocal interaction, association with clinical and pathological characteristics, and prognosis in Caucasian patients with operable NSCLC.

Materials and Methods

Patients

The study group comprised 208 patients with NSCLC who underwent curative pulmonary resection at the Thoracic Surgery Department of the Medical University of Gdańsk between 2005 and 2008 (Table 1). The median age of this group was 62 years (range: 41–80 years). The most common type of surgery was lobectomy (148 patients, 71.2%), followed by pneumonectomy (35 patients, 16.8%). Bilobectomy, segmentectomy, wedge resection, and sleeve lobectomy were performed in 13 (6.3%), 8 (3.8%), 2 (1%), and 2 (1%) patients, respectively. All patients underwent mediastinal lymphadenectomy. Four patients (1.9%) received induction chemotherapy, 10 (4.8%) received adjuvant chemotherapy, and 1 (0.5%) received postoperative radiotherapy. A total of 108 (51.9%), 97 (46.6%), and 3 (1.4%) patients were diagnosed with squamous cell lung carcinoma, adenocarcinoma, and large cell carcinoma, respectively. Most of the patients were diagnosed with stage I and II NSCLC according to the Seventh Edition of the tumor, node, metastasis (TNM) Classification of Malignant Tumors (Table 1). Initial staging included bronchofiberoscopy, computed tomography of the chest and upper abdomen, and, depending on the clinical situation, ultrasound-guided endobronchial biopsy and mediastinoscopy. Over the study period, however, positron emission tomography was not routinely used as a staging procedure. Patient data were retrieved from the clinical records.

This study was approved by the Independent Bioethics Committee of the Medical University of Gdańsk.

Table 1. Patient Characteristics.

Characteristics	N	(%)
Age, years		
Median	62	
Range	41–80	
Sex		
Female	68	32.7
Male	140	67.3
Smoking status		
Smoker	129	62.0
Nonsmoker	79	38.0
Histological type		
Squamous	108	51.9
Adenocarcinoma	97	46.6
Large cell carcinoma	3	1.4
Pathological stage		
IA	38	18.3
IB	58	27.9
IIA	45	21.6
IIB	28	13.5
IIIA	39	18.8
IIIB	0	0.0
IV	0	0.0
Grade		
G1	11	5.3
G2	140	67.3
G3	57	27.4

Tissue Microarray

Two representative archival core tissue sections (0.6 mm in diameter) were taken from each of the 416 paraffin "donor" blocks containing postoperative primary tumor samples and manually arranged into tissue microarrays (TMAs) using a Manual Tissue Arrayer (Beecher Instruments; Inc., Sun Prairie, WI).[19] Four core tissue sections retrieved from different cell-rich cancer regions (identified and marked by an experienced pathologist) were analyzed for each case to compensate for possible cancer heterogeneity. The sampled cores were then allocated into 12 new "recipient" paraffin blocks. Next, TMAs were cut into 4-μm sections using a microtome, mounted on FLEX IHC Microscope Slides (Dako; Santa Clara, CA), and incubated at 37C overnight. A dedicated TMA map was prepared to assist in reading out the IHC staining results.

IHC Assays

First, tissue sections from the TMA blocks were deparaffinized in xylene and rehydrated in graded ethanol.

Next, epitopes were uncovered with a heat-induced epitope retrieval procedure using a Dako PT Link apparatus with an EnVision FLEX Target Retrieval Solution (Dako). The expression of the study proteins was evaluated using IHC. First, all sections were incubated with primary antibodies:

- Rabbit polyclonal antibody for Wnt1 (#PA5-32641; dilution 1:100, positive control/breast cancer; Thermo Scientific Pierce, Waltham, MA)
- Rabbit monoclonal antibody for Wnt2 (#3169-1, EPR3101[2] clone; dilution 1:350, positive control/fetal lung; Epitomics, Boston, MA)
- Mouse monoclonal antibody for β-catenin (#M3539, β-catenin-1 clone; dilution 1:200, positive control/colon cancer; Dako)
- Mouse monoclonal antibody for E-cadherin (#IR059, NCH-38 clone; dilution 1:100, positive control/palatine tonsil epithelium; Dako)

Subsequently, they were exposed to a secondary antibody (Envision Rabbit/Mouse), stained using a diaminobenzidine detection kit, and counterstained with hematoxylin. The IHC staining for Wnt1, Wnt2, β-catenin, and E-cadherin was evaluated under a light microscope (Olympus BX43; Warsaw, Poland) for every core tissue section by two independent observers: an experienced pathologist and a clinician (A.W.) who completed dedicated training. The evaluation included the cytoplasmic expression of Wnt1 and Wnt2; the membranous, diffuse cytoplasmic, and nuclear expression of β-catenin; and the cytoplasmic and membranous expression of E-cadherin.

The IHC reactions were manually and semi-quantitatively assessed using the *H*-score, taking into account the percentage of stained cells, staining intensity (0–3), and staining distribution: *H*-score = [1x (% of cells stained at intensity category 1+) + 2x (% of cells stained at intensity category 2+) + 3x (% of cells stained at intensity category 3+)].[20] An *H*-score between 0 (no staining) and 300 was obtained, with 300 representing 100% of tumor cells stained strongly (3+). The highest *H*-score calculated for the four core sections representing each case was included in the statistical analysis. The median *H*-scores for particular proteins were used as cut-off values for the interpretation of stainings as positive: 175 for Wnt1, 100 for cytoplasmic β-catenin, and 200 for Wnt2, E-cadherin, and nuclear and membranous β-catenin (Fig. 1).

Statistical Analysis

The median value and range were used for descriptive analyses. Associations between continuous variables were tested using a nonparametric test for independent samples (Mann–Whitney *U* or Kruskal–Wallis test). Categorized variables were compared using the χ^2 test. Pearson's correlation coefficient was used to analyze the correlation between two continuous variables. A *p* value less than 0.05 was considered statistically significant. Survival curves were computed using the Kaplan–Meier analysis. Overall survival (OS) was defined as the time from surgery to the date of death or the last follow-up visit. Recurrence-free survival (RFS) was defined as the time from surgery to the date of confirmed local or regional recurrence, distant spread, or death from any cause or the date of the last follow-up visit. A log-rank test and Cox proportional hazards regression model were used in univariate and multivariate analyses to evaluate whether a biomarker represents an independent prognostic factor for OS. All *p* values were based on two-sided statistical analyses, and *p* values below 0.05 were considered to indicate statistical significance without multiple comparison adjustment. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 22.0; IBM Corp., Armonk, NY).

Results

The median follow-up time, defined as the time from the date of surgery to the last follow-up visit or death, was 68 months (range: 0.2–117 months). The mean effective follow-up time for the analyzed cohort was 58.9 months (4.9 years). The median survival in the entire group was not reached and the 5-year OS probability was 58% (95% CI: 0.514–0.654). In total, 30 patients (14.4%) developed local recurrence, 30 (14.4%) developed regional recurrence, and 34 (16.3%) developed distant metastases. The most common metastatic sites included the brain (30 patients, 14.4%), bones (16 patients, 7.7%), and liver (6 patients, 2.9%). By the time of the analysis, 82 patients (39.4%) were deceased and 23 (11.1%) were lost to follow-up.

Wnt1 expression was found in 103 cases (49.5%, Table 2), and the median and mean *H*-scores for Wnt1 protein expression were found to be 175 (range: 0–300) and 162.5 (±59.8), respectively. The calculated mean Wnt1 *H*-scores for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma were 148 (±53), 178 (±63), and 183 (±76), respectively. Wnt1 expression was significantly lower in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma (*p*<0.0001, Table 3), for G3 compared with G1/G2 tumors of all analyzed lung cancer histological subtypes (*p*=0.003) as well as for IIB/IIIA compared with IA/IIA pathological stages (*p*<0.042, Table 3). No significant correlations were found for Wnt1 expression
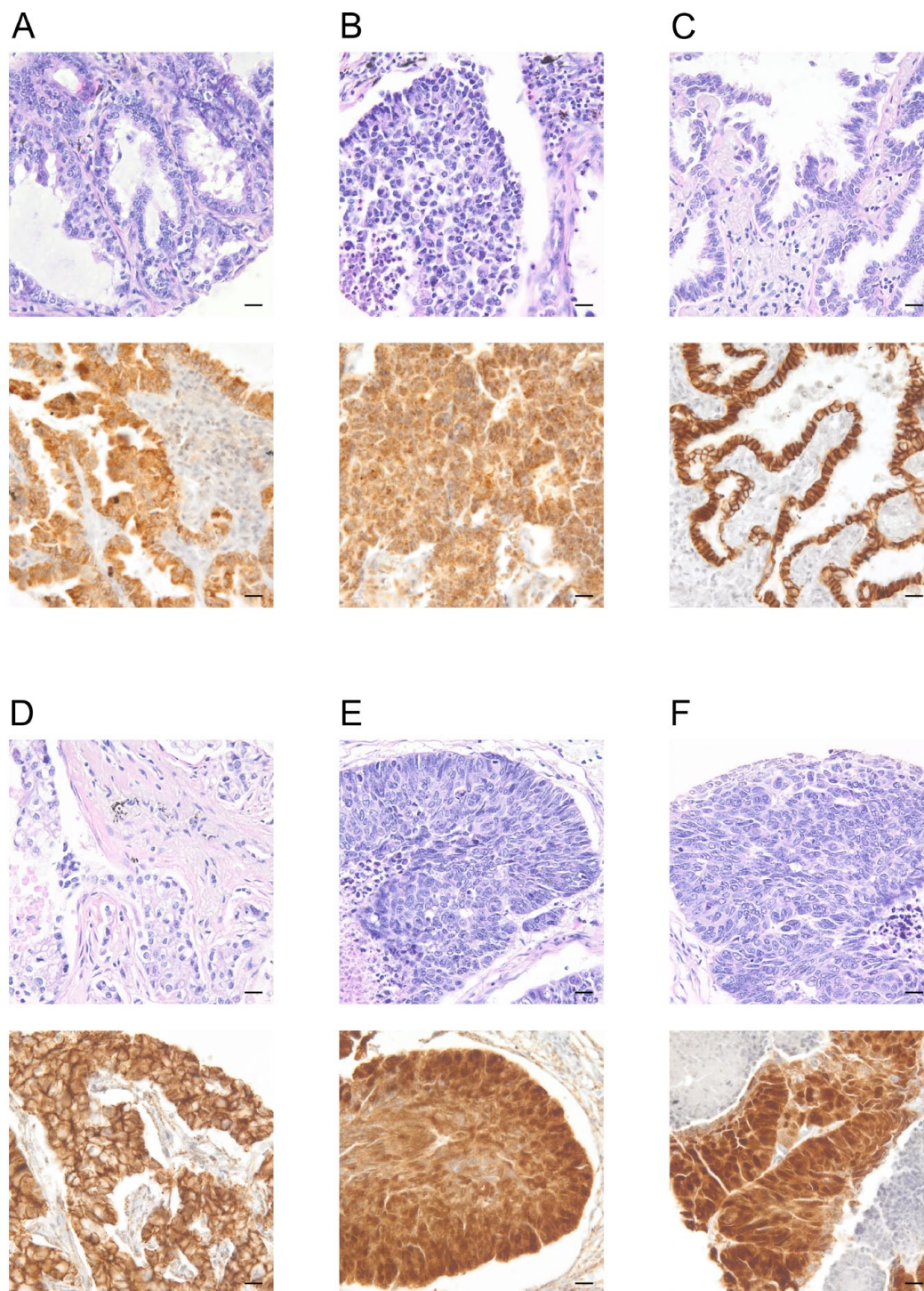
Figure 1. Immunohistochemical staining patterns of Wnt1, Wnt2, E-cadherin, and β-catenin in operable non-small cell lung cancer accompanied by corresponding H&E images (upper row). Scale bar = 20 μm. (A) Cytoplasmic expression of Wnt1 (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300. (B) Cytoplasmic expression of Wnt2 (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300. (C) Membranous/cytoplasmic expression of E-cadherin (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300. (D) Membranous expression of β-catenin (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300. (E) Cytoplasmic expression of β-catenin (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300. (F) Nuclear expression of β-catenin (40× eyepiece magnification multiplied by 10× objective lens magnification; total magnification, 400×); *H*-score = 300.

Table 2. Expression of Wnt1, Wnt2, E-Cadherin, and β-catenin Fractions.

Characteristics	Wnt1	Wnt2	E-Cadherin	Nuclear β-catenin	Membranous β-catenin	Cytoplasmic β-catenin
Cases stained positive % (*n*)	49.5 (103)	22.3(46)	37.4 (77)	1.2 (3)	23.7 (49)	34.8 (72)
Median *H*-score	175	200	200	NA	200	100
Mean *H*-score	162.5	203.62	207	NA	178	104
H-score for SCC histology	148	195	190	NA	174	174
H-score for AC histology	178	212	224	NA	185	185
H-score for LCC histology	183	233	236	NA	123	123

Abbreviations: SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.

Table 3. Correlation Between Wnt1, Wnt2, E-Cadherin, and β-catenin Expression and Clinicopathological Characteristics (Univariate Analysis; p Values).

Variable	Wnt1	Wnt2	E-Cadherin	Nuclear β-catenin	Membranous β-catenin	Cytoplasmic β-catenin
Age	0.216	0.565	0.452	0.123	0.590	0.726
Sex	0.577	0.971	0.099	0.215	0.661	0.371
Smoking status	0.625	0.719	0.103	0.293	0.514	0.855
Histological type	**≤0.0001**	0.124	**0.001**	0.777	0.435	0.264
T status	0.363	0.767	0.778	0.795	0.670	0.379
N status	0.056	0.305	0.165	0.737	0.778	0.939
Pathological stage	**0.042**	0.739	0.470	0.824	0.272	0.407
Grade	**0.003**	**0.002**	**0.010**	0.084	**0.008**	0.922
Primary tumor diameter	**0.027**	0.569	0.339	0.437	0.145	0.118
Tumor necrosis	**0.002**	0.772	0.099	0.411	0.055	0.073
Pleural invasion	0.312	0.284	**0.039**	0.305	0.618	0.141

Significant values are marked in bold.

with age, sex, smoking status, T and N stages, or pleural invasion (*p*>0.05). However, a weak inverse correlation was observed between Wnt1 expression and primary tumor diameter ($r = -0.154$, *p*=0.027) and tumor necrosis ($r = -0.212$, *p*=0.002).

Wnt2 expression was found in 46 cases (22.3%, Table 2), and the median and mean *H*-scores for Wnt2 protein expression were 200 (range: 90–300) and 203.62 (±44.39), respectively. The calculated mean Wnt2 *H*-scores for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma were 195 (±46), 212 (±40), and 233 (±57), respectively. The median value of Wnt2 expression was significantly lower in G3 than in G1/G2 tumors of all analyzed lung cancer histological subtypes (*p*=0.002, Table 3). No significant correlations were found for Wnt2 expression with age, sex, smoking status, tumor histology, T and N stages, primary tumor diameter, tumor necrosis, pathological stage, or pleural invasion (*p*>0.05).

E-cadherin expression was found in 77 cases (37.4%, Table 2), and the median and mean *H*-scores of E-cadherin protein expression were 200 (range: 10–300) and 207 (±62), respectively. The mean E-cadherin

H-scores for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma were 190 (±58), 224 (±61), and 236 (±55), respectively. The median value of E-cadherin expression was significantly lower in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma (*p*=0.001, Table 3), for G3 compared with G1/G2 tumors of all analyzed lung cancer histological subtypes (*p*=0.01) as well as for tumors with pleural involvement (*p*=0.039). No significant correlations were found for E-cadherin expression with age, sex, smoking status, T and N stages, pathological stage, primary tumor diameter, or tumor necrosis. However, a weak positive correlation was observed between E-cadherin and Wnt2 protein expression ($r = 0.151$, *p*=0.03), and a relatively strong positive correlation was observed between E-cadherin and membranous β-catenin expression ($r = 0.569$, *p*=0.0001).

Nuclear expression of β-catenin was found in 3 cases (1.2%, Table 2), and membranous expression of β-catenin was found in 49 cases (23.7%). The median and mean *H*-scores for the membranous fraction of β-catenin were 200 (range: 0–300) and 178 (±78), respectively. The calculated mean membranous β-catenin

Table 4. Recurrence-free Survival According to Clinicopathological Characteristics (Multivariate Cox Analysis).

Variable	HR	95% CI	*p* value
Age	0.994	0.969–1.019	0.629
Sex (female vs. male)	1.094	0.671–1.784	0.719
Smoking status (smoker vs. nonsmoker)	1.141	0.719–1.811	0.576
Tumor histology (SCC vs. AC vs. LCC)	1.031	0.654–1.625	0.896
Primary tumor diameter	1.187	1.09–1.28	**<0.0001**
Primary tumor necrosis rate	1.001	0.99–1.01	0.85
Pleural invasion	1.622	1.27–2.05	**<0.0001**
T feature	1.46	1.21–1.74	**<0.0001**
N feature	2.357	1.82–3.03	**<0.0001**
Pathological stage	1.750	1.478–2.073	**<0.0001**
Wnt1 expression	1.000	0.996–1.004	0.997
Wnt2 expression	0.999	0.994–1.004	0.722
E-cadherin expression	0.999	0.995–1.003	0.750
Nuclear β-catenin expression	1.002	0.997–1.008	0.412
Membranous β-catenin expression	0.998	0.995–1.001	0.159
Cytoplasmic β-catenin expression	1.001	0.998–1.004	0.543

Values in boldface are statistically significant (*p*<0.05). Abbreviations: CI, confidence interval; HR, hazard ratio; SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.

H-scores for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma were 174 (±80), 185 (±74), and 123 (±157), respectively. The expression of membranous β-catenin was significantly higher in G1 than in G2/G3 tumors of all analyzed lung cancer histological subtypes (*p*=0.008, Table 3). However, no significant associations were observed between the expression of membranous β-catenin and clinical and pathological features.

Cytoplasmic β-catenin expression was found in 72 cases (34.8%, Table 2), and the median and mean *H*-scores for the cytoplasmic fraction of β-catenin were 100 (range: 0–300) and 104 (±84), respectively. The calculated mean cytoplasmic β-catenin *H*-scores for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma were 174 (±80), 185 (±74), and 123 (±157), respectively. A weak positive correlation was observed between the membranous and cytoplasmic expression of β-catenin (*r* = 0.328, *p*=0.0001). However, no significant correlation was found between the expression of cytoplasmic β-catenin and clinicopathological variables (Table 3).

In the multivariate analysis, RFS and OS were related to the T status (*p*<0.0001 for both), tumor diameter (*p*<0.0001), pleural invasion (*p*=0.001), N status (*p*<0.0001), and stage (*p*<0.0001, Tables 4 and 5). A significant association was found between OS and tumor grade (*p*<0.023). However, univariate Cox analysis showed that Wnt1 does not correlate with RFS or OS [*p*=0.265, hazard ratio (HR) = 0.998 and *p*=0.164, HR=0.997, respectively]. No correlation was observed between RFS and OS and the expression of Wnt2

(*p*=0.503, HR = 0.998 and *p*=0.695, HR=0.999, respectively) and cytoplasmic β-catenin (*p*=0.481, HR = 0.999 and *p*=0.451, HR = 0.999, respectively). The expression of E-cadherin did not correlate with RFS (*p*=0.102, HR = 0.997). However, lower E-cadherin expression was significantly associated with shorter OS (*p*=0.039, HR = 0.996). Notably, a significant association was observed between RFS and OS and the expression of membranous β-catenin (*p*=0.006, HR = 0.997 and *p*=0.007, HR = 0.996, respectively). In a multivariate Cox analysis adjusted for clinical and pathological features, no significant association was found between RFS and OS and the expression of the study proteins.

Discussion

This is the first study evaluating the expression of two Wnt family proteins (Wnt1, Wnt2), E-cadherin, and β-catenin, as well as their interaction, association with pathological and clinical features, and prognostic significance in Caucasian patients with NSCLC. In this study, cytoplasmic Wnt1 expression was found in half of the NSCLC cases, in line with the results of earlier studies, which have all been performed in Asia.[21–23] However, in their study, Xu et al.[23] used a different *H*-score cut-off (>100) to define positive staining. Moreover, another two studies did not report the staining methodology or cut-off values. The expression of Wnt1 was found to be significantly lower in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma. These results are in line with those of Xu

Table 5. Overall Survival According to Clinicopathological Characteristics (Multivariate Cox Analysis).

Variable	HR	95% CI	*p* value
Age	1.002	0.976–1.029	0.876
Sex (female vs. male)	1.084	0.643–1.827	0.761
Smoking status (nonsmoker vs. smoker)	0.942	0.586–1.513	0.805
Tumor histology (SCC vs. AC vs. LCC)	1.069	0.657–1.740	0.788
Primary tumor diameter	1.183	1.08–1.28	**<0.0001**
Primary tumor necrosis rate	0.999	0.98–1.01	0.91
Pleural invasion	1.523	1.18–1.96	<0.001
T feature	1.479	1.21–1.79	**<0.0001**
N feature	2.233	1.71–2.91	**<0.0001**
Pathological stage	1.642	1.379–1.953	**<0.0001**
Wnt1 expression	0.999	0.995–1.003	0.676
Wnt2 expression	1.001	0.996–1.006	0.757
E-cadherin expression	0.998	0.994–1.003	0.476
Nuclear β-catenin expression	1.002	0.997–1.008	0.419
Membranous β-catenin expression	0.998	0.995–1.001	0.225
Cytoplasmic β-catenin expression	1.001	0.998–1.004	0.632

Values in boldface are statistically significant (*p*<0.05).Abbreviations: CI, confidence interval; HR, hazard ratio; SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.

et al.,[23] indicating the potentially greater importance of Wnt1 pathway activation in adenocarcinoma, although no such correlation has been observed in the two abovementioned studies.[21,22] This discrepancy may be due to differences in the staining methodology and cut-offs adopted.

In this study, the median Wnt1 expression was found to be significantly lower in poorly differentiated than in well and moderately differentiated tumors. We hypothesize that a higher grade may be associated with different posttranslational protein alterations, modulation of Wnt pathway inhibitor activity (i.e., WIF-1, DKK-3) associated with epigenetic mechanisms (DNA methylation and histone modification), activation of Wnt1-independent ligands, and cross talk with other molecular pathways (i.e., MET- and IGF-dependent) inhibiting Wnt-dependent activation. This correlation, however, was not analyzed by Xu et al.[23] and was not found in another two studies in Asia, possibly because of the different IHC staining cut-offs adopted.[21–23]

In contrast to the abovementioned studies in Asia,[21–23] the median expression of Wnt1 in our study was found to be lower at the pIIB/pIIIA stages than at the IA–IIA stages. It was also observed that the distribution of lung cancer stages in our study is similar to that in the cited studies, precluding potential bias. However, the lower Wnt1 expression observed in more advanced disease may suggest increased Wnt1 activity at earlier stages of carcinogenesis.

Similar to the study of Xu et al.,[23] we found a weak inverse correlation for Wnt1 expression with primary tumor diameter and the presence of tumor necrosis.

Extensive necrosis in cancer is typical in less differentiated and larger tumors, and lower expression of Wnt1 in more advanced and less differentiated tumors corresponds to its lower level in necrotic cancers. Because the TMA technology uses samples of selected tumor regions, we retrieved four core tissue sections to compensate for the tumor heterogeneity. Thus, the result of the inverse correlation between tumor diameter and Wnt1 may mirror higher Wnt1 expression at earlier stages of carcinogenesis, which decreases with tumor growth. However, we found no significant associations between the expression of Wnt1 and age, sex, smoking status, T and N stages, or pleural invasion. These results are in line with those of the three abovementioned studies in Asia.[21–23] The positive correlation found in our study between Wnt1 and Wnt2 expression may suggest a sibling function of these proteins and their engagement on the same level of the canonical Wnt pathway. Such an association between Wnt1 and Wnt2 has not been analyzed before.

In this study, cytoplasmic Wnt2 expression was found in 22% of the cases. However, Huang et al.[24] reported a higher rate of Wnt2 expression (63%), likely because of the different cut-offs adopted in that study or the potential genotypic differences between Asian and Caucasian populations.

Similar to Wnt1, the expression of Wnt2 was significantly lower in poorly differentiated tumors than in more differentiated ones (well and moderately differentiated). It was also found that the expression of Wnt2 is not associated with the age, sex, smoking status, tumor histology, T and N stages, tumor necrosis, pathological

stage, or pleural invasion. However, Huang et al.[24] reported higher Wnt2 expression in higher grade adenocarcinomas. These discrepancies may be explained by the higher proportion of adenocarcinomas and stage III cancers in that study, different cut-offs, and the potential genotypic, transcriptomic, proteomic, and metabolomic differences between Asian and Caucasian populations.

In this study, membranous and/or cytoplasmic E-cadherin expression was found in 37% of the cases. These data are in line with the results of two studies in Asia.[25,26] Similar to other studies,[25,27,28] the expression of E-cadherin was significantly lower in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma. This finding suggests more pronounced adhesion disorders in squamous cell lung cancers, as well as their stronger tendency for local invasion, and the significant role of E-cadherin loss in the development of this NSCLC type. Similar to earlier studies,[25,27,29–31] the expression of E-cadherin was found in this study to be significantly lower in poorly than in well-differentiated tumors. Hence, we hypothesize that a lower E-cadherin level may be associated with a loss in the differentiation potential, leading to more pronounced adhesion disorders in lung cancers with higher grades. Generally, lower E-cadherin expression stimulates the endothelial-mesenchymal transition (EMT) of cancer cells,[25,27,29,30,31] enabling their dissociation and local invasion. Although our results are in line with those of two other studies,[32,33] they are discordant with a meta-analysis published by Yang et al.[31] Moreover, in line with earlier studies,[30,31,34,35] we found no correlation between the expression of E-cadherin and age, sex, smoking status, T and N stages, pathological stage, primary tumor diameter, or tumor necrosis. However, the weak positive correlation observed in our study between E-cadherin and Wnt2 protein expression may be explained by the association among bifunctional β-catenin, E-cadherin–catenin unit (ECCU), and Wnt-dependent canonical pathway. Similar to earlier studies,[25,30] in this study, a relatively strong positive correlation was observed between E-cadherin and β-catenin expression. In general, higher grade lung cancers are characterized by ECCU dysfunction, which results in a simultaneous decrease in the expression of E-cadherin and β-catenin. Notably, low expression of membranous β-catenin, as well as its presence in the cytoplasmic compartment and nuclear translocation, is interpreted as a disrupted expression pattern of this protein. Membranous and cytoplasmic expression of β-catenin was found in 24% and 35% of the cases, respectively, whereas β-catenin nuclear expression was sporadic. A large discrepancy has been observed in the β-catenin staining interpretation; for example,
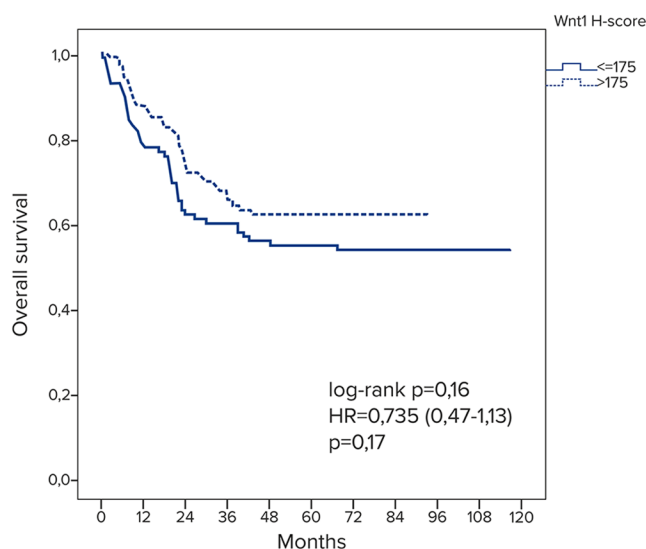

Figure 2. Overall survival of operable NSCLC patients in relation to the Wnt1 status. Abbreviations: HR, hazard ratio; NSCLC, non-small cell lung cancer.

some authors have interpreted membranous staining as negative.[36,37] Notably, most studies have assessed the general cellular β-catenin expression, without dividing it into particular compartments.[23,32,38,39] This lack of standardization in IHC staining and its interpretation hinders comparisons between these studies. In our series, the expression of membranous β-catenin expression was significantly lower in G3 than in G1/G2 tumors of all analyzed lung cancer histological subtypes (p=0.008). Lower β-catenin concentrations generally disrupt cellular differentiation and promote local invasion. Hence, our results are in line with previously published studies[25,32,40] but are discordant with the meta-analysis of Yang et al.[31]

No significant correlations were observed for membranous and cytoplasmic β-catenin expression with clinical and pathological features. These data are in line with the results of the abovementioned meta-analysis.[31] A weak positive correlation was observed between membranous and cytoplasmic expression of β-catenin, which may be explained by the disruption of β-catenin expression associated with its migration from the cell membrane to the cytoplasm and further translocation into the nucleus. Such a correlation, however, has not been analyzed earlier.

Our study confirms the prognostic significance of well-known factors: T status, tumor diameter, pleural invasion, N status, and tumor stage and grade. Unlike in the studies in Asia,[21–24] the expression of Wnt1 and Wnt2 was not predictive of RFS or OS (Figs. 2 and 3). These differences may be due to the distinct features between Asian and Caucasian populations,
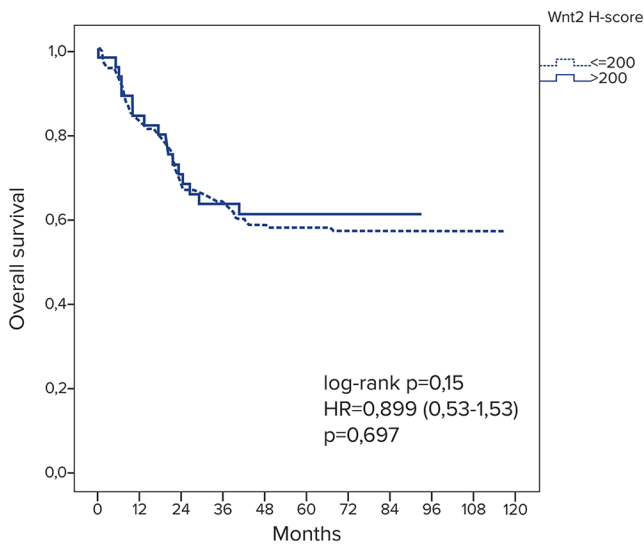
Figure 3. Overall survival of operable NSCLC patients in relation to the Wnt2 status. Abbreviations: HR, hazard ratio; NSCLC, non-small cell lung cancer.
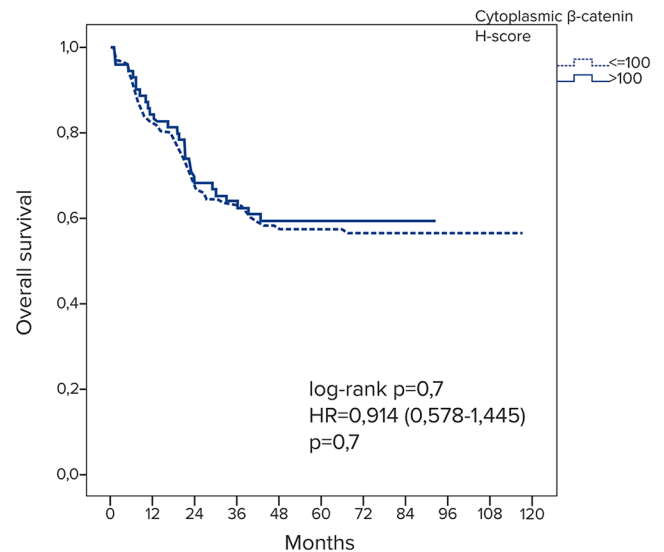

Figure 5. Overall survival of operable NSCLC patients in relation to the cytoplasmic β-catenin status. Abbreviations: HR, hazard ratio; NSCLC, non-small cell lung cancer.
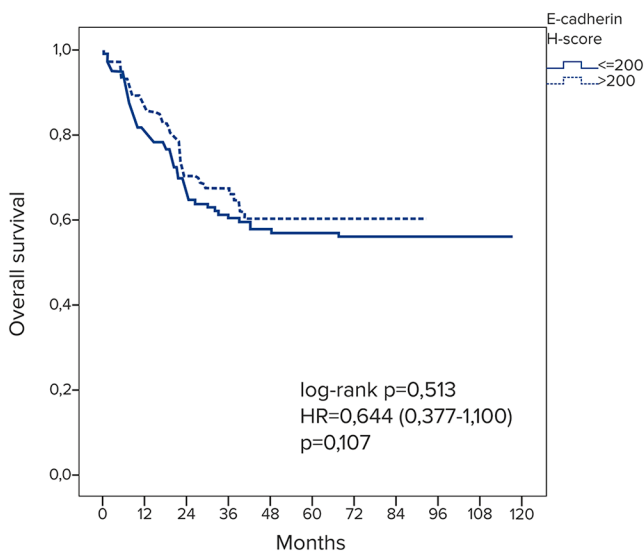

Figure 4. Overall survival of operable NSCLC patients in relation to the E-cadherin status. Abbreviations: HR, hazard ratio; NSCLC, non-small cell lung cancer.
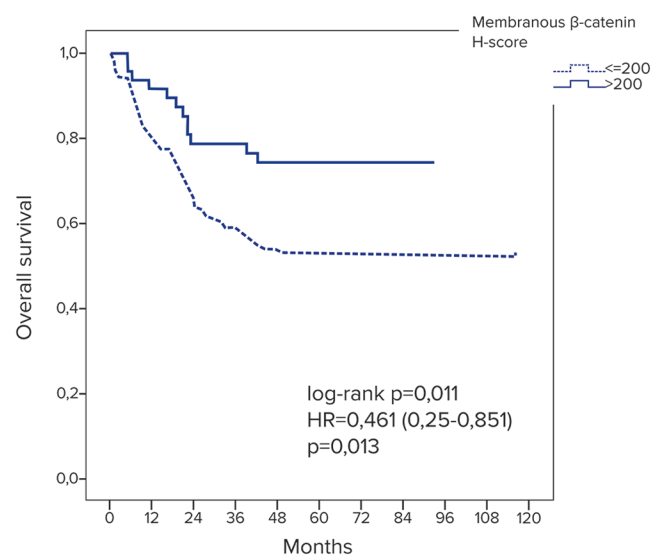

Figure 6. Overall survival of operable NSCLC patients in relation to the membranous β-catenin status. Abbreviations: HR, hazard ratio; NSCLC, non-small cell lung cancer.

the different antibodies used for IHC staining, and the different cut-offs.

In the univariate analysis, lower E-cadherin expression was associated with shorter OS. This finding is in line with previously published data.[27,28,30,31,34] In a multivariate Cox analysis adjusted for clinical and pathological features, no significant association was found between the expression of E-cadherin and RFS or OS (Fig. 4). This finding is in line with the results of three earlier studies[25,28,30] but discordant with those of the two other

studies.[33,41] A lower E-cadherin concentration leads to the impairment of adhesive interactions and promotes stromal and angiogenic invasion, consequently leading to metastasis.[42] Similar to previously published data,[30] in this study, no association was found between OS or RFS and the expression of cytoplasmic β-catenin (Fig. 5). Nuclear β-catenin expression was found only in three cases, precluding any statistical analysis.

In this study, lower membranous β-catenin expression was associated with shorter RFS and OS in the

univariate analysis. Low expression of β-catenin generally leads to the impairment of adhesive interactions and promotes stromal and angiogenic invasion, hence inducing metastasis. However, similar to the results of two earlier studies,[40,41] the expression of membranous β-catenin was found to be not predictive of RFS or OS in the multivariate Cox analysis (Fig. 6). However, other studies focusing on EMT have shown the prognostic value of this variable.[25,36,37] As explained above, the lack of standardization in IHC staining of β-catenin and its interpretation limits comparisons between studies.

The results of this study suggest the activation of the canonical Wnt-dependent signaling pathway in NSCLC and its role in the different steps of cancer induction and promotion. These may include the stimulation of proliferation and neoangiogenesis, inhibition of cancer cell apoptosis, EMT induction, stromal invasion, and development of chemoresistance and radioresistance. However, the interpretation of the significance of the Wnt signaling pathway in lung cancer oncogenesis is difficult because of the complex interplay between Wnt proteins and the regulatory impact of noncanonical pathways (other pathways activated by Wnt proteins, either polar or calcium-dependent). There are also numerous canonical pathway inhibitors and epigenetic mechanisms underlying their inactivation (mainly hypermethylation promotors of genes encoding inhibitors, e.g., WIF-1, axin, SFRP-1, Dkk-5, and DACT2), as well as a cross talk with other pathways (dependent on HGF, EGF, TGFβ, VEGF, FGF, and GF activation, as well as Notch, Hedgehog, PG/Cox-2, PI3K/AKT, mTOR, Jak-STAT, and Ras).[9] The activity of the Wnt pathway should be carefully interpreted in a broader molecular context, including all the interactions mentioned above in the simulating models. In colon cancer, deregulation of the Wnt pathway results in higher accumulation of the β-catenin–lymphoid enhancer factor/T-cell factor (LEF/TCF) complex in the cytoplasm and nucleus, leading to hereditary colon cancer and familial adenomatous polyposis.[43,44] However, these findings should not be extrapolated to other malignancies, including NSCLC, as the final effect of this pathway's activation strongly depends on the tissue context. Adequate interpretation of events at the functional level seems to be crucial, as the presence of a protein should not be treated as a surrogate of its active function.

Activation of the Wnt pathway stimulates the renewal of cancer stem cells and induces chemoresistance and radioresistance. Development of Wnt inhibitors and their combinations with chemoresistance and radiotherapy may potentially improve the efficacy of treatment by either delaying or overcoming the mechanisms of resistance.

In summary, this study provides novel data on several key proteins involved in the Wnt signaling pathway in NSCLC and may serve as background information for further molecular and functional studies. However, it should be noted that none of the analyzed proteins carries prognostic information in operable lung cancer in Caucasian populations, and hence they cannot be used in selecting high-risk patients for adjuvant therapies.

Competing Interests

Author Contributions

AW: Conception, design, and planning of the study. Statistical calculations. Analysis of the data and interpreting the results. Drafting of the manuscript and critically reviewing or revising the manuscript for important intellectual content. Interpretation of immunohistochemical stainings (second observer). RD and JJ: Drafting of the manuscript and critically reviewing or revising the manuscript for important intellectual content. AS: Interpretation of immunohistochemical stainings (first observer), photographs preparation. All authors have read and approved the final manuscript.

Funding

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