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EGFR Level in Non-Small Cell Lung Cancers Is Associated with the Expression of SATB1 and EMT-Promoting Factors

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Abstract: Epidermal Growth Factor Receptor (EGFR) expression is an important aspect in the non-small cell lung cancer (NSCLC) diagnosis and treatment. Therefore, it becomes important to identify factors that may influence EGFR level. There are reports suggesting that one of the positive EGFR gene transcription regulators may be Special AT-rich Binding Protein 1 (SATB1), but so far these observations have not been confirmed in NSCLC. **Aim of the study:** The main aim of the present study was to investigate the possible links between the EGFR and SATB1 expression on both protein and mRNA levels in NSCLC clinical samples and to correlate the obtained results with the clinical-pathological data of the patients. Additionally, we analyzed the relationships between the expression levels of EGFR and the known tumor promoters, including Ki67 proliferative antigen and EMT-promoting transcription factors (SLUG, SNAIL, and Twist1). **Materials and methods:** The study was conducted on 239 NSCLC clinical samples. The methods used included immunohistochemistry and chromogenic *in situ* hybridization (CISH). **Results and conclusions:** We demonstrated that EGFR expression in NSCLC was positively associated both with the SATB1 level and with the expression of EMT-promoting proteins. Moreover, we were the first to analyze EGFR expression exclusively in NSCLC cancer cells without the interference caused by respiratory epithelium and tumor stroma. Our analysis revealed that the prognostic significance of EGFR expression was dependent on tumor histology and differed significantly between the AC and LSCC samples.

Keywords: SATB1; EGFR; NSCLC; AC; LSCC; non-small cell lung carcinoma; lung adenocarcinoma; lung squamous cell carcinoma; epithelial growth factor receptor

1. Introduction

Lung carcinoma is the most commonly diagnosed cancer worldwide and the leading cause of cancer-related mortality [1]. Lung cancers can be classified into four primary categories: adenocarcinomas (ACs), squamous cell lung carcinomas (LSCCs), large cell carcinomas (LCCs), and small cell lung cancers (SCLC) [2]. ACs, LSCCs, and LCCs are often collectively classified as non-small cell lung cancers (NSCLCs). Although NSCLC is not included in the official histological classification, the term is widely used in clinical practice, with tumors classified as NSCLCs accounting for nearly 90% of all lung cancer cases [3]. This study will focus exclusively on NSCLCs, with particular emphasis on the AC and LSCC subtypes. Due to its relatively low incidence and high heterogeneity, LCC will not be included in this analysis.

The most commonly used targeted therapies for NSCLC are epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs). EGFR is a transmembrane protein that can be

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activated by ligands from the epidermal growth factor (EGF) family [4]. Upon activation, EGFR undergoes dimerization, which triggers tyrosine kinase-mediated signal transduction through the RAS/RAF/MAPK, PI3K/AKT, or JAK/STAT pathways. EGFR activation leads to increased cell proliferation and migration, as well as resistance to apoptosis [4]. EGFR is expressed in approximately 50% of NSCLC cases [5]; however, not all EGFR-positive tumors are eligible for EGFR-TKI treatment. The most favorable responses to EGFR-TKIs have been observed in patients with "activating" or "sensitizing" mutations in the EGFR kinase domain [4]. These mutations include deletions in exon 19 and the Leu858Arg substitution in exon 21 [5], which result in constitutive, ligand-independent activation of the receptor.

In its official guidelines, the American Society of Clinical Oncology recommends the use of EGFR-TKIs exclusively for patients with sensitizing mutations [6]. However, there are reports indicating that a positive response to EGFR-TKIs can also occur in patients with elevated levels of wild-type EGFR [7,8]. Given this information, it is crucial to identify factors that may act as regulators of EGFR expression, as they could serve as potential molecular markers or targets for novel therapies. It is known that the transcription of the *EGFR* gene can be regulated by the Sp1 and c-Jun proteins [9]. However, there are reports suggesting that another *EGFR* transcription regulator may be Special AT-rich Binding Protein 1 (SATB1) [10].

SATB1 is a nuclear matrix protein often referred to as a "global transcription factor." It organizes DNA into tertiary structures by binding to specific genomic sequences known as BURs (base-unpairing regions) (*base-unpairing regions*) [11]. By recruiting additional transcription factors and chromatin modifying enzymes, SATB1 simultaneously regulates the expression of entire sets of genes, including those located on distant chromosomes [11]. Physiologically, SATB1 is involved in the proliferation, differentiation, and migration of cells, regulating gene expression in a tissue-specific manner [11]. In cancer cells, the presence of SATB1 has been associated with an aggressive phenotype and resistance to apoptosis [12]. Furthermore, SATB1 has been shown to play a role in the epithelial-mesenchymal transition process, thereby contributing to cancer metastasis [12]. Overexpression of SATB1 has been demonstrated to have negative prognostic significance in breast, gastrointestinal, ovarian, and prostate cancers [12,13].

In NSCLC, the role of SATB1 expression has been observed to be strictly dependent on the tumor histology. In AC, elevated SATB1 levels were associated with a poor degree of tumor differentiation [14,15]. In LSCC in turn, a reverse relationship has been noticed - SATB1 expression was negatively associated with the tumor grade [15]. In NSCLC samples, SATB1 level was found to be associated with the expression of the proliferative marker Ki67 [15] and EMT-promoting transcription factors [16]. Moreover, SATB1 expression has been demonstrated to be a positive prognostic factor for NSCLC and LSCC patients [16,17]. In AC, its prognostic significance has not been confirmed.

It is known that there are positive associations between SATB1 level and EGFR expression. In 2008, Han *et al*. demonstrated that in breast cancer cells, SATB1 upregulates the expression of *EGFR* and other genes involved in EGF signaling [10]. In glioblastoma cell lines, SATB1 knockdown negatively influenced the expression levels of *EGFR* and other proto-oncogenes [18]. A similar effect of *SATB1* knockdown was noticed also in colon cancer cell lines [19]. However, there are no reports available about SATB1/EGFR relationships in NSCLC. The aim of the presented study was to investigate the possible links between SATB1 and EGFR levels in NSCLC clinical samples and to correlate the obtained results with the clinical-pathological data of the patients.

4. Materials and Methods

Patient Cohort

The study group consisted of 239 patients treated in the Lower Silesian Centre of Lung Diseases in Wroclaw during the years 2007-2016. A total of 239 NSCLC samples were collected during planned surgical procedures and preserved in formalin-fixed paraffin-embedded (FFPE) blocks. The study was approved by the Bioethics Commission at the Wroclaw Medical University in Poland, approval no. 632/2017. The clinical-pathological data of the patients are listed in **Table 1** and **Table 2**.

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Table 1. Clinical-pathological data of the patients. IHC studies. AC, adenocarcinoma; LSCC, squamous cell carcinoma. Age is expressed in years, all other data are expressed as n (%).

Parameters		**All cases (N=239) n (%)**	**AC (N=149) n (%)**	**LSCC (N=90) n (%)**
Gender	Male	144 (60.2)	85 (57.0)	59 (65.6)
	Female	95 (39.8)	64 (43.0)	31 (34.4)
Age (years)	Median	66	65	66
	Range	44-84	44-84	44-82
Malignancy grade	G1	3 (1.3)	3 (2.0)	0 (0.0)
	G2	149 (62.3)	72 (48.3)	77 (85.6)
	G3	87 (36.4)	74 (49.7)	13 (14.4)
Tumor size	pT1	75 (31.4)	56 (37.6)	19 (21.1)
	pT2	122 (51.0)	65 (43.6)	57 (63.3)
	pT3	23 (9.6)	11 (7.4)	12 (13.3)
	pT4	5 (2.1)	4 (2.7)	1 (1.1)
	No data	23 (9.6)	13 (8.7)	1 (1.1)
Lymph nodes	pN0	144 (60.2)	82 (55.0)	62 (68.9)
	pN1	40 (16.7)	23 (15.4)	17 (18.9)
	pN2	41 (17.2)	31 (20.8)	10 (11.1)
	No data	14 (5.9)	13 (8.7)	1 (1.1)
Stage	I	102 (42.7)	64 (43.0)	38 (42.2)
	II	76 (31.8)	36 (24.2)	40 (44.4)
	III	45 (18.8)	34 (22.8)	11 (12.2)
	IV	2 (0.83)	2 (1.3)	0 (0.0)
	No data	14 (5.9)	13 (8.7)	1 (1.1)
Overall survival	Deaths	94 (39.3)	62 (41.6)	32 (35.6)
	Alive	144 (60.3)	86 (57.7)	58 (64.4)
	No data	1 (0.42)	1 (0.67)	0 (0.0)

Table 2. Clinical-pathological data of the patients. CISH studies. AC, adenocarcinoma; LSCC, squamous cell carcinoma. Age is expressed in years, all other data are expressed as n (%).

Parameters		**All cases (N=170) n (%)**	**AC (N=104) n (%)**	**LSCC (N=66) n (%)**
Gender	Male	104 (61.18)	58 (55.77)	46 (69.70)
	Female	66 (38.82)	46 (44.23)	20 (30.30)
Age (years)	Median	66	66	66
	Range	44-82	44-82	52-82
Malignancy grade	G1	1 (0.59)	1 (0.96)	0 (0.0)
	G2	105 (61.76)	49 (47.11)	56 (84.85)
	G3	64 (37.65)	54 (51.92)	10 (15.15)
Tumor size	pT1	51 (30.00)	36 (34.61)	15 (22.73)

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	pT2	87 (51.18)	47 (45.19)	40 (60.61)
	pT3	16 (9.41)	6 (5.77)	10 (15.15)
	pT4	5 (2.94)	4 (3.85)	1 (1.51)
	No data	11 (6.47)	11 (10.58)	0 (0.0)
Lymph nodes	pN0	105 (61.76)	58 (55.77)	47 (71.21)
	pN1	23 (13.53)	12 (11.54)	11 (16.67)
	pN2	31 (18.24)	23 (22.11)	8 (12.12)
	No data	11 (6.47)	11 (10.58)	0 (0.0)
Stage	I	70 (41.18)	45 (43.27)	25 (37.88)
	II	53 (31.18)	21 (20.19)	32 (48.48)
	III	34 (20.00)	25 (24.03)	9 (13.64)
	IV	2 (1.18)	2 (1.92)	0 (0.0)
	No data	11 (6.47)	11 (10.58)	0 (0.0)
Overall survival	Deaths	100 (58.82)	42 (40.38)	39 (59.09)
	Alive	69 (40.59)	61 (58.65)	27 (40.91)
	No data	1 (0.59)	1 (0.96)	0 (0.0)

Tissue Microarrays (TMAs)

Tissue microarrays (TMAs) were prepared as previously described [16].

Immunohistochemistry (IHC)

Immunohistochemical reactions were performed on 4-µm-thick paraffin sections using DAKO Autostainer Link48 (Dako; Agilent Technologies, Inc.) and EnVision FLEX reagents (Dako; Agilent Technologies, Inc.) according to the manufacturer's instructions. Primary anti-EGFR antibodies (cat. no. M7239; Clone E30; Dako; Agilent Technologies, Inc.) were diluted 1:50 and applied for 20 min at RT.

IHC staining for SATB1, Ki67, SLUG, SNAIL, Twist1, N-cadherin, and E-cadherin proteins were performed previously, and the methodology was described in [15] and [16].

Evaluation of the IHC Stainings

IHC slides were evaluated using the QuantCenter (3DHistech) software as previously described [15,16]. Membranous expression of EGFR was assessed using the scale ranging from 0 to 3, based on the percentage of positive cells and the reaction intensity (**Table 3**).

Table 3. Scoring system used for the evaluation of EGFR stainings.

Score	Percentage of the positive cells and intensity of the staining
0	No staining is observed or staining is observed in <10% of the tumor cells
1	A faint membrane staining is observed in >10% of the tumor cells
2	A weak or moderate, complete membrane staining is observed in >10% of the tumor cells
3	A strong, complete membrane staining is observed in >10% of the tumor cells

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Chromogenic In Situ Hybridization (CISH)

All of the CISH reagents were components of the ViewRNA™ Tissue Core Kit (Invitrogen, Waltham, MA, USA), unless stated otherwise. CISH was performed on 4-µm thick paraffin sections cut from the TMAs. To deparaffinize the sections, Histo-Clear (National Diagnostics, Atlanta, GA, USA) was used. After the deparaffinization, the slides were washed twice with 100% ethanol and allowed to dry. Then, the slides were immersed in 1x Pretreatment Solution and incubated for 10 minutes in 95°C. To increase the target's accessibility, protease digestion (20 min, 40°C) was performed. Finally, the sections were fixed for 5 minutes with the 10% solution of the neutral buffered formalin (Sigma-Aldrich, Saint Louis, MO, USA) in PBS (Sigma-Aldrich) and stored overnight at 4°C, immersed in PBS.

The next day, target probe sets were hybridized. The probes used are listed in the **Table 4**. 18S ribosomal RNA was used as an endogenous control, to ensure the RNA integrity. The robes were prepared according to the manufacturer's instructions, then the slides were placed in the DAKO Hybridizer (Agilent, Santa Clara, CA, USA) and hybridization was carried out for 2 hours at 40°C. Subsequently, sections were washed thoroughly in Wash Buffer. After that, the Label Probe 1 – AP hybridization (15 min, 40°C) and Fast Red staining (30 min, RT) were performed. Finally, all the sections were counterstained with Gill's Hematoxylin No. 1 (Sigma-Aldrich) for 5 minutes at room temperature, allowed to dry, and mounted with ADVANTAGE Mounting Medium (Innovex Biosciences, Richmond, CA, USA).

Table 4. Probes used for the CISH reactions.

Target molecule	Gene name	Probe number
mRNA for EGFR protein	*EGFR*	VA1-11736-VT (Thermo Fisher Scientific)
mRNA for SATB1 protein	*SATB1*	VA1-13726-VT (Thermo Fisher Scientific)
18S ribosomal RNA	*555RN18S1*	VA1-3020734-VT (Thermo Fisher Scientific)

Evaluation of the CISH Slides

Obtained CISH slides were scanned with the Pannoramic MIDI II scanner and subjected to further digital analysis. To precisely count red dots representing specific mRNA molecules, QuPath [20] software was used. First, the specimens were pre-segmented using a pixel classifier. Then, cancer cells were identified and counted. Finally, individual red dots were counted using an experimental "Subcellular detection" algorithm. Dot clusters were digitally separated into individual particles. The final result is given as the average number of specific mRNA molecules/cancer cell. Our methodology was based on the QuPath analysis guidelines published on the ACD website [21].

Statistical Analysis

The obtained results were analyzed using Prism 8.0 (GraphPad Software, La Jolla, CA, USA) and Statistica 13 (StatSoft, Krakow, Poland) statistical software. Shapiro-Wilk test was utilized to determine whether the sample data were normally distributed. To compare the groups of data, a non-parametric Mann-Whitney U test was used. Correlations between the analyzed parameters were verified using Spearman's rank correlation test. Survival times were determined by the Kaplan-Meier method, and the significance of the differences was determined by a log-rank test. All the results were considered statistically significant when the $p<0.05$.

2. Results

EGFR Protein Expression Was Significantly Higher in LSCC Compared to AC Tumors

To assess EGFR protein levels in the analyzed clinical samples, IHC staining was performed. EGFR expression was observed in the nuclei, cytoplasm, and membranes of cancer cells (**Figure 1 A-C**), and

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in the non-malignant lung epithelium (**Figure 1 D**). No EGFR staining was detected in the tumor stroma, non-malignant lung alveoli (**Figure 1 E**), or in the infiltrating lymphocytes (**Figure 1 F**).

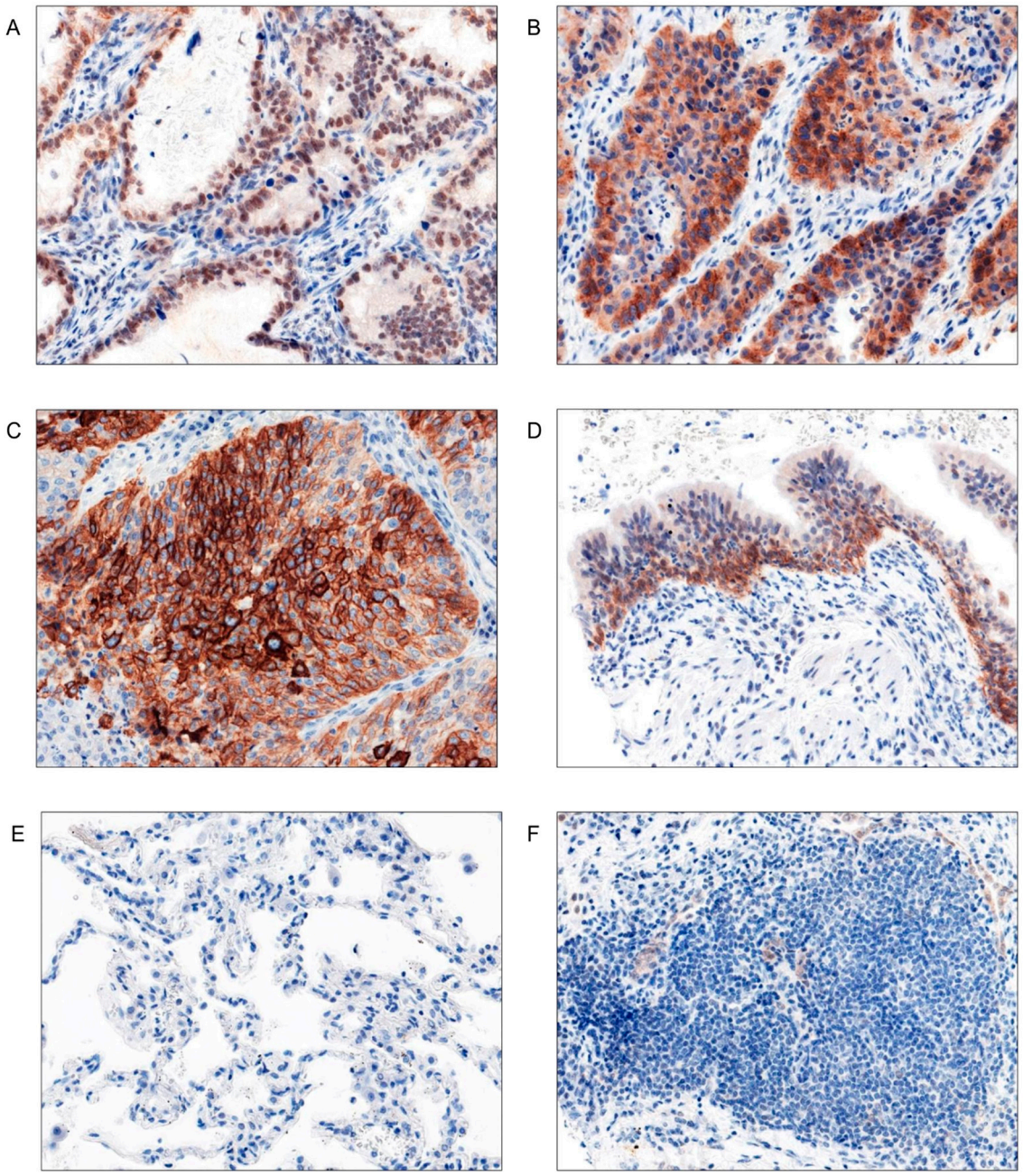

Figure 1. Immunohistochemical staining for EGFR expression in non-small cell lung cancer specimens and non-malignant lung tissues. Cell nuclei are stained blue, brown color indicates a positive immunohistochemical reaction for EGFR protein. EGFR was expressed in the nuclei (A), cytoplasm (B), and membranes (C) of cancer cells. EGFR expression was also observed in the basal layer of non-malignant respiratory epithelium (D). There was no EGFR expression in the non-malignant lung pneumocytes (E) or infiltrating lymphocytes (F).

We noticed nuclear EGFR expression (score>3) in 90 of 239 (37.66%) of the analyzed NSCLC samples. Further statistical analysis revealed that the mean EGFR N score values were significantly higher in LSCC than in AC tumors (3.53±0.84 vs. 3.11±0.99; $p<0.001$; **Figure 2 A**). No additional associations between EGFR N expression and clinical-pathological data of the patients were observed (**Table S1**).

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EGFR was expressed in the cytoplasm of cancer cells in 139 of 239 (58.16%) cases. As in the case of EGFR N expression, EGFR C scores were significantly higher in LSCC compared to AC samples (4.56±1.36 vs. 3.66±1.17; p<0.001; **Figure 2 B**). Moreover, in AC tumors analyzed separately, we observed an association between EGFR C expression and the tumor grade – the expression was significantly higher in G3 tumors in comparison to G1 and G2 ones (3.88±1.22 vs. 3.45±1.08; p=0.03; **Figure 2 D**; **Table S2**).

Membranous EGFR staining was present in 26 out of 239 (10.88%) of the samples. The mean scores were significantly higher in LSCC in comparison to AC specimens (0.41±0.87 vs. 0.09±0.41; p<0.001; **Figure 2 C**; **Table S3**).

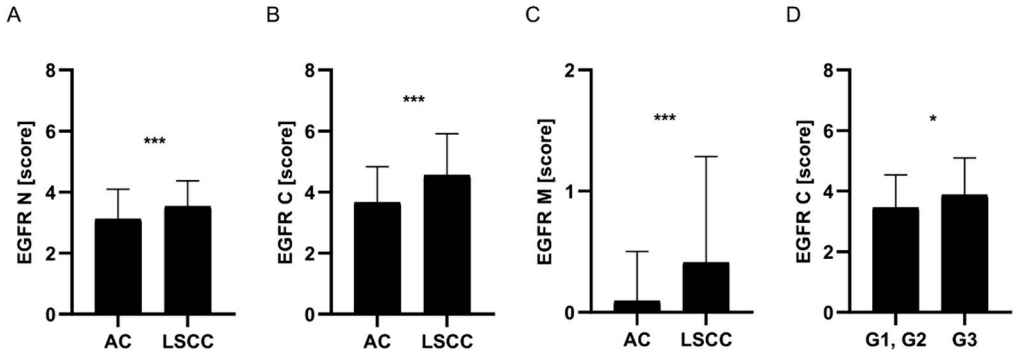

Figure 2. EGFR protein expression in non-small cell lung cancer specimens. Immunohistochemical staining for EGFR protein, followed by digital analysis of obtained slides, revealed that EGFR expression was significantly higher in the nuclei (**A**), cytoplasm (**B**), and membranes (**C**) of LSCC cells when compared to AC samples. Moreover, in AC specimens we observed an association between EGFR expression and tumor grade – EGFR scores were significantly higher in G3 compare to G1 and G2 tumors combined (**D**). **AC** - adenocarcinoma; **LSCC** - squamous cell carcinoma; * - p≤0.05; *** - p≤0.001.

EGFR Protein Expression in Cancer Cells Was Positively Correlated with SATB1 Protein Level and with the Expression of EMT-Related Transcription Factors

We observed a significant positive association between nuclear EGFR protein expression and SATB1 level in cancer cells. This relationship was present both in the whole NSCLC cohort (R=0.504; p≤0.0001), and in the AC and LSCC subtypes analyzed separately (R=0.464; p≤0.0001 and R=0.431; p≤0.0001, respectively; **Figure 3 A-C**). A similar relationship was also noticed between the expression of EGFR and SLUG in cancer cell nuclei. The correlation between the expression of these factors had the highest statistical significance in AC tumors (R=0.337; p≤0.0001; **Figure 3 E**), but was present also in the whole NSCLC cohort (R=0.343; p≤0.01; **Figure 3 D**), and the LSCC subtype (R=0.214; p≤0.05; **Figure 3 F**). Nuclear EGFR staining was positively correlated also with SNAIL and cytoplasmic SLUG expression in NSCLC and AC, but not in the LSCC subgroup (correlation coefficients and p values can be found in **Table 5**). Moreover, nuclear EGFR level positively correlated with Twist1 expression in NSCLC and LSCC but not in AC (**Table 5**). We also observed a positive correlation between nuclear EGFR and Ki67 expression, but it was present only in the whole NSCLC cohort (**Table 5**).

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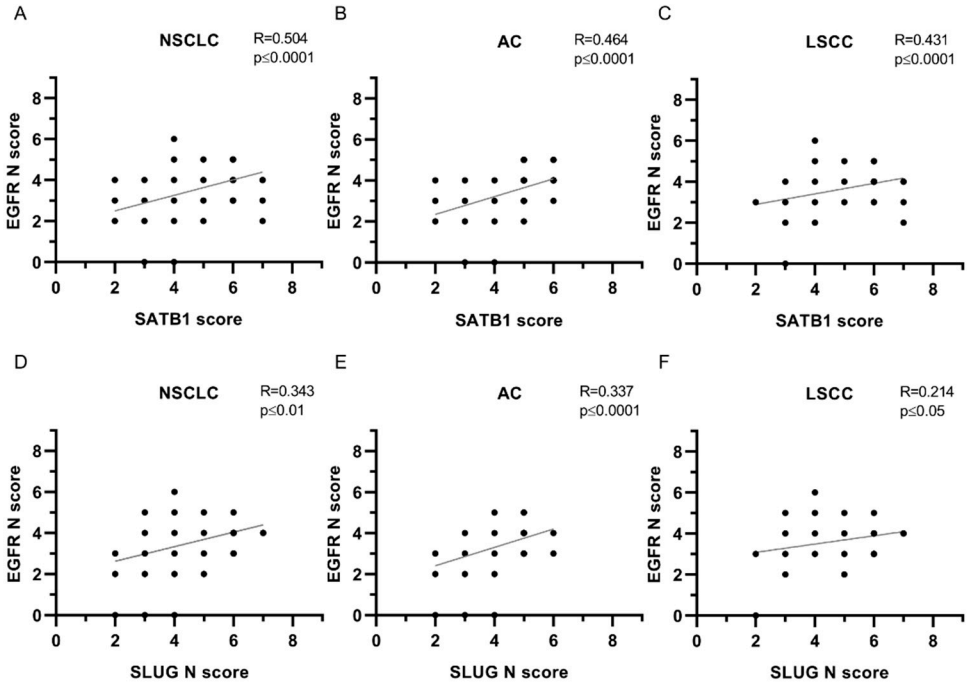

Figure 3. Associations between the expression levels of EGFR, SATB1, and SLUG proteins in non-small cell lung cancer specimens. EGFR expression in cancer cells nuclei was positively associated with SATB1 level in NSCLC (A), AC (B), and LSCC (C) samples. Moreover, nuclear EGFR scores were associated positively also with nuclear SLUG level in NSCLC (D), AC (E), and LSCC (F) specimens. NSCLC – non-small cell lung carcinoma; AC - adenocarcinoma; LSCC – squamous cell carcinoma.

Table 5. Correlations between the nuclear expression of EGFR and expression of SATB1, Ki67, E-cadherin, N-cadherin, SNAIL, SLUG , and Twist1 proteins. Significant P-values are given in bold. Ns – non-significant; * - p≤0.05; ** - p≤0.01; *** - p≤0.001; **** - p≤0.0001.

EGFR N						
Protein	**NSCLC**		**AC**		**LSCC**	
	Spearman's R	P-value	Spearman's R	P-value	Spearman's R	P-value
SATB1	**0.504**	****	**0.464**	****	**0.431**	****
Ki67	**0.206**	***	0.106	ns	0.186	ns
E-cadherin	0.043	ns	0.004	ns	0.088	ns
N-cadherin	0.035	ns	-0.013	ns	0.052	ns
SNAIL	**0.129**	*	**0.205**	*	-0.086	ns
SLUG N	**0.343**	**	**0.337**	****	**0.214**	*
SLUG C	**0.173**	****	**0.227**	**	0.160	ns
Twist1	**0.249**	***	0.156	ns	**0.241**	*

EGFR expression observed in cancer cells' cytoplasm was positively correlated with SATB1 level in the whole NSCLC cohort and in the AC subgroup (R=0.387; p≤0.0001 and R=0.336; p≤0.0001, respectively; **Figure 4 A** and **Figure 4 D**). A similar relationship was noticed also in LSCC tumors, but it was less significant (R=0.233, p≤0.05; **Figure 4 G**). Cytoplasmic EGFR level positively correlated also with the nuclear and cytoplasmic expression of SLUG. This correlation was observed in all of the analyzed groups: in NSCLC (R=0.347; p≤0.0001 for SLUG N; **Figure 4 B**; R=0.226; p≤0.001 for SLUG C), AC (R=0.248; p≤0.01 for SLUG N; R=0.203; p≤0.05 for SLUG C), and LSCC (R=0.257; p≤0.05 for

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SLUG N; **Figure 4 H**; R=0.363; p≤0.001 for SLUG C; **Figure 4 I**). In NSCLC and AC, we also noticed a positive correlation between the expression of EGFR C, SNAIL, and Twist1 proteins (**Table 6**). Moreover, cytoplasmic EGFR immunostaining was positively associated with Ki67 expression, but this relationship was present only in the whole NSCLC cohort (**Table 6**).

The expression of EGFR observed in the membranes of cancer cells was only slightly associated with the expression of the other analyzed factors. The correlation between EGFR M level and SATB1 expression was present only in the whole NSCLC cohort (R=0.166; p≤0.001), just like the correlation with SLUG N level (R=0.184; p≤0.001). There was also a weak positive correlation between EGFR M immunostaining and Ki67 expression, observed both in the whole NSCLC cohort and AC subtype (**Table 7**), and a weak correlation between EGFR M and SNAIL in the AC subgroup (**Table 7**).

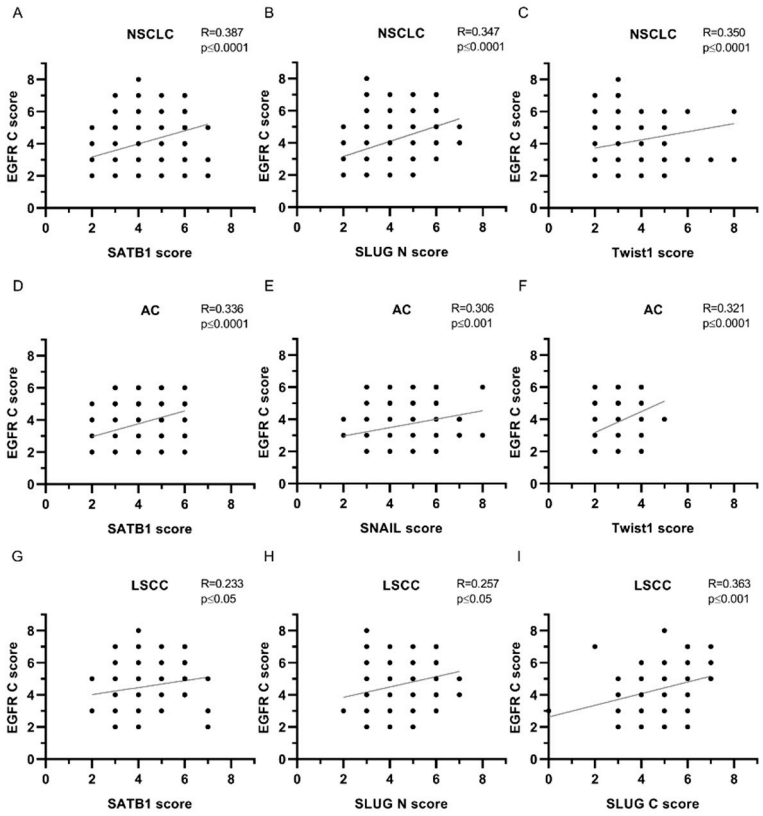

Figure 4. Associations between the expression levels of EGFR, SATB1, and EMT-promoting proteins in non-small cell lung cancer specimens. EGFR expression in cancer cells cytoplasm was positively associated with SATB1 level in NSCLC (A), AC (D), and LSCC (G). Moreover, cytoplasmic EGFR scores were associated positively with nuclear SLUG level in NSCLC (B) and LSCC (H). We observed also positive associations between EGFR C score and Twist1 score (C, F), EGFR C score and SNAIL score (E), and EGFR C score and SLUG C score (I). NSCLC – non-small cell lung carcinoma; AC - adenocarcinoma; LSCC – squamous cell carcinoma.

Table 6. Correlations between the cytoplasmic expression of EGFR and expression of SATB1, Ki67, E-cadherin, N-cadherin, SNAIL, SLUG , and Twist1 proteins. Significant p-values are given in bold. Ns – non-significant; * - p≤0.05; ** - p≤0.01; *** - p≤0.001; **** - p≤0.0001.

EGFR C						
Protein	**NSCLC**		**AC**		**LSCC**	
	Spearman's R	P-value	Spearman's R	P-value	Spearman's R	P-value
SATB1	**0.387**	****	**0.336**	****	**0.233**	*
Ki67	**0.217**	***	0.084	ns	0.117	ns

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E-cadherin	0.071	ns	0.015	ns	0.132	ns
N-cadherin	0.039	ns	-0.138	ns	0.118	ns
SNAIL	**0.220**	***	**0.306**	***	0.008	ns
SLUG N	**0.347**	****	**0.248**	**	**0.257**	*
SLUG C	**0.226**	***	**0.203**	*	**0.363**	***
Twist1	**0.350**	****	**0.321**	****	0.151	ns

Table 7. Correlations between the membranous expression of EGFR and expression of SATB1, Ki67, E-cadherin, N-cadherin, SNAIL, SLUG , and Twist1 proteins. Significant p-values are given in bold. Ns – non-significant; * - p≤0.05; ** - p≤0.01; *** - p≤0.001; **** - p≤0.0001.

EGFR M						
Protein	**NSCLC**		**AC**		**LSCC**	
	Spearman's R	P-value	Spearman's R	P-value	Spearman's R	P-value
SATB1	**0.166**	**	0.044	ns	0.137	ns
Ki67	**0.186**	**	**0.206**	*	0.027	ns
E-cadherin	0.065	ns	0.053	ns	0.053	ns
N-cadherin	0.093	ns	0.072	ns	0.065	ns
SNAIL	0.093	ns	**0.204**	*	-0.031	ns
SLUG N	**0.184**	**	0.102	ns	0.135	ns
SLUG C	0.080	ns	0.063	ns	0.142	ns
Twist1	0.075	ns	0.009	ns	-0.041	ns

The Prognostic Significance of EGFR mRNA Expression Depended on Tumor Histology

EGFR mRNA expression was assessed using the CISH technique. This method made it possible to investigate the *EGFR* mRNA level specifically in cancer cells with no interference caused by *EGFR* expression in the respiratory epithelium. *EGFR* mRNA was detected in 88/239 (36.82%) of the analyzed specimens (**Figure 5 A**). We observed no association between the number of *EGFR* mRNA copies per cancer cell and tumor histology, grade, size, patients' lymph node status or stage of the disease (**Table S1**).

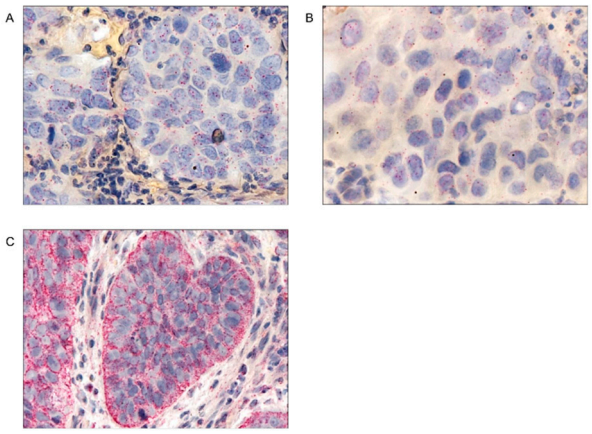

Figure 5. *EGFR* mRNA (A), *SATB1* mRNA (B), and 18S RNA (C) expression in non-small cell lung cancer cells, assessed using CISH technique. Each red dot represents a target RNA molecule. Cell nuclei are stained blue.

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To determine the impact of *EGFR* expression on patients' survival, Kaplan-Meier's survival curves were compared using the log-rank (Mantel-Cox) test. The results obtained revealed that *EGFR* mRNA expression showed no association with patients' survival in the whole NSCLC cohort. However, statistically significant differences in survival curves were observed in AC and LSCC subtypes analyzed separately. In AC tumors, high *EGFR* mRNA expression (>0.24 mRNA copies/cell) was associated with significantly better patients' survival (p=0.015; **Figure 7 A**), whereas in LSCC tumors, high *EGFR* mRNA levels (>0.05 mRNA copies/cell) were associated with poor prognosis (p=0.046; **Figure 7 B**).

Expression of SATB1 mRNA Increased with Patients' Age

We observed *SATB1* mRNA expression in 51/239 (21.34%) of the investigated NSCLC cases (**Figure 5 B**). The further analysis of the obtained results revealed a significant link between *SATB1* mRNA expression and the patients' age. The number of *SATB1* mRNA copies per cancer cell was significantly higher in patients over the age of 65 compared to those aged 65 and less. The described relationship was present in the whole NSCLC cohort (0.13±0.44 vs. 0.51±0.88; p<0.001; **Figure 6 A**), and in the AC subtype analyzed separately (0.09±0.34 vs. 0.46±0.79; p<0.001; **Figure 6 B**). In the LSCC subtype, the differences were on the verge of statistical significance (0.20±0.58 vs. 0.59±1.01; p=0.06). No other associations between the *SATB1* mRNA level and clinical-pathological data of the patients were noticed.

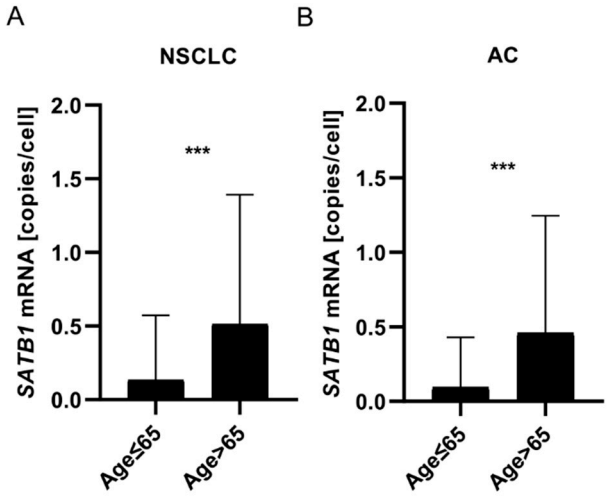

Figure 6. *SATB1* mRNA expression was associated with patients age. After performing CISH assays, followed by digital analysis of SATB1 mRNA expression in lung cancer cells, we observed that SATB1 mRNA level was significantly higher in patients over the age of 65 compared to those aged 65 and younger. This relationship was present both in the whole NSCLC cohort (**A**) and in the AC subtype analyzed separately (**B**). **NSCLC** – non-small cell lung carcinoma; **AC** - adenocarcinoma; *** - p≤0.001.

SATB1 mRNA Expression Was Associated with Better Prognosis for NSCLC Patients

To determine the impact of SATB1 expression on patients' survival, Kaplan-Meier's survival curves were compared using the log-rank (Mantel-Cox) test. The results obtained revealed that high *SATB1* mRNA expression (more than 2 *SATB1* mRNA copies per cancer cell) was associated with a significantly better patients' prognosis (p=0.012; **Figure 7 C**). Additionally, we observed a trend towards improved survival in AC patients with *SATB1* expression higher than 1 mRNA copy per cancer cell (p=0.062).

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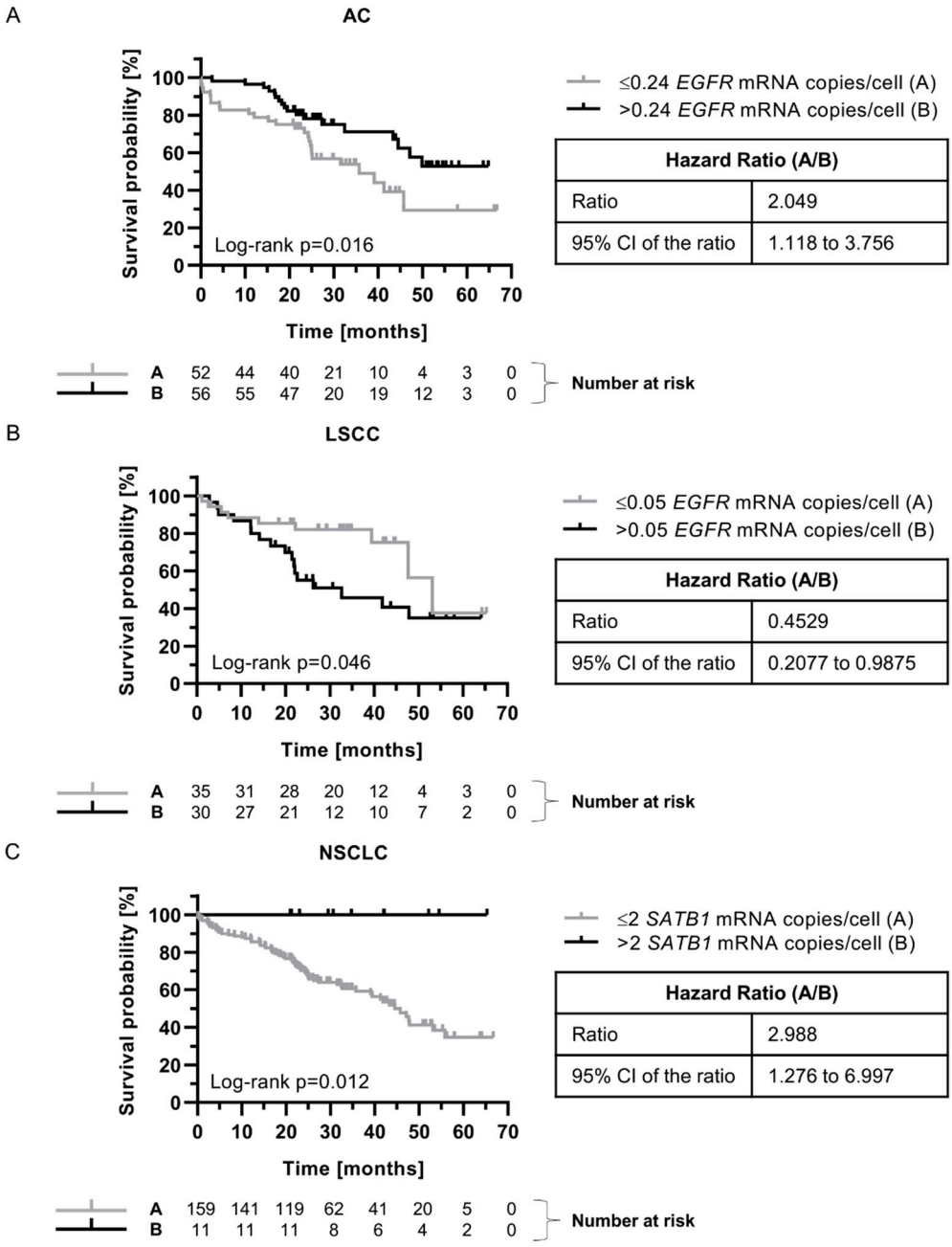

Figure 7. The prognostic significance of *EGFR* mRNA expression in non-small cell lung cancer specimens was dependent on the tumor histology. EGFR mRNA expression analysis in lung cancer cells revealed that high EGFR mRNA copy number (>0.24 copies/cell) was a positive prognostic factor for AC patients (A). On the contrary, for LSCC patients, prognosis was significantly better when EGFR mRNA copy number was low (≤0.05 mRNA copies/cell) (B). High SATB1 mRNA expression (more than 2 mRNA copies/cell) was also associated with significantly better patients' prognosis (C). NSCLC – non-small cell lung carcinoma; AC - adenocarcinoma; LSCC – squamous cell carcinoma.

EGFR mRNA Expression Was Negatively Associated with the Level of EGFR Protein and EMT-Promoting Transcription Factors

EGFR mRNA level, assessed specifically in the cancer cells with the use of the CISH method, was negatively associated with nuclear and cytoplasmic EGFR protein levels. These relationships were present both in the whole NSCLC cohort (R=-0.196; p≤0.01 for EGFR N; R=-0.195; p≤0.01 for EGFR C), and in the AC subtype (R=-0.345; p≤0.001 for EGFR N; R=-0.260; p≤0.01 for EGFR C). Moreover, *EGFR* expression correlated negatively with the SLUG N level in all of the analyzed groups: NSCLC (R=-0.251; p≤0.001), AC (R=-0.247; p≤0.01), and LSCC (R=-0.260; p=0.05). There were

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also negative correlations between *EGFR* mRNA level and the expression of Twist1 in NSCLC and AC, and between *EGFR* and SNAIL in NSCLC (**Table 8**). We did not observe any association between *EGFR* mRNA expression and the expression of *SATB1* mRNA or SATB1 protein.

Table 8. Correlations between the expression of *EGFR* mRNA and expression of SATB1, Ki67, E-cadherin, N-cadherin, SNAIL, SLUG , and Twist1 proteins. Significant p-values are given in bold. Ns – non-significant; * - p≤0.05; ** - p≤0.01; *** - p≤0.001; **** - p≤0.0001.

***EGFR* mRNA**						
Protein	**NSCLC**		**AC**		**LSCC**	
	Spearman's R	P-value	Spearman's R	P-value	Spearman's R	P-value
EGFR N	**-0.196**	**	**-0.345**	***	0.094	ns
EGFR C	**-0.195**	**	**-0.260**	**	-0.088	ns
EGFR M	-0.081	ns	0.008	ns	-0.149	ns
***SATB1* mRNA**	-0.078	ns	-0.226	ns	-0.092	ns
SATB1	-0.131	ns	-0.170	ns	-0.073	ns
Ki67	-0.021	ns	0.075	ns	-0.153	ns
E-cadherin	-0.011	ns	0.054	ns	-0.180	ns
N-cadherin	-0.128	ns	-0.079	ns	-0.165	ns
SNAIL	**-0.185**	*	-0.133	ns	**-0.283**	*
SLUG N	**-0.251**	***	**-0.247**	**	**-0.260**	*
SLUG C	-0.118	ns	-0.076	ns	-0.188	ns
Twist1	**-0.183**	*	**-0.280**	**	-0.048	ns

3. Discussion

The importance of EGFR expression in NSCLC results not only from the oncogenic function of this receptor but also from its role as a significant therapeutic target. Unfortunately, although effective, therapy with EGFR-TKIs has some serious limitations. First, only the patients with specific EGFR mutations are sensitive to EGFR inhibition. Second, over time, all of them develop resistance to EGFR-TKIs. For these reasons, there is an ongoing search for proteins that could potentially regulate EGFR expression and serve as targets for new therapies. In our study, we focused on the SATB1 protein as a potential EGFR expression regulator. SATB1 is a potent transcriptional factor with a known ability to influence *EGFR* transcription in breast cancer and glioblastoma cells. However, there are no reports about its possible impact on EGFR level in NSCLC.

In our study, we observed EGFR expression in the nuclei, cytoplasm, and membranes of cancer cells. This may seem surprising because EGFR is commonly thought to be a strictly membranous protein. However, the possibility of its nuclear translocation has been known for over 30 years—nuclear EGFR expression was first observed in human adrenocortical carcinoma in 1990 [22]. Since then, nuclear EGFR staining has been described in a wide variety of samples, including both normal tissues and malignant tumors [23]. In the cell nucleus, EGFR functions as a transcriptional co-activator, influencing the expression of several oncogenes related to cell proliferation, angiogenesis, and therapy resistance [23,24]. Nuclear EGFR has been described as a negative prognostic factor in numerous tumors, including breast, ovary, oropharynx, and laryngeal cancers [23].

In NSCLC, nuclear EGFR expression was previously observed both in AC and LSCC tumors [25,26]. In our study, we noticed significantly higher EGFR N scores in LSCC compared to AC samples. This is in good agreement with Traynor *et al*., who also reported a higher percentage of cells expressing EGFR N in LSCC when compared to AC specimens [26]. However, they observed an association between EGFR N expression and a higher stage of the disease, whereas our results showed no significant relationship between EGFR N level and clinical-pathological data of the

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patients. We have also observed no impact of EGFR N expression on patients' survival. Traynor *et al*., on the contrary, revealed that EGFR N overexpression was associated with a shorter overall survival of the patients [26]. On the other hand, Wang *et al*., in their study on AC tumors, found that EGFR N expression was significantly associated with the recurrence risk but not with mortality [25]. These discrepancies are probably due to the differences in the analyzed patient cohorts—our study included NSCLC patients regardless of the stage of the disease, while Traynor *et al.*'s study group consisted of early-stage NSCLC tumors, and Wang *et al*. analyzed only AC samples.

In our study, we observed elevated EGFR levels in the cytoplasm of LSCC cells when compared to AC samples. Cytoplasmic EGFR expression was previously described in numerous malignancies, including lung, rectal, head and neck, and non-melanoma skin cancers [24,27–29]. In NSCLC, cytoplasmic EGFR staining was noticed by several researchers [27,30,31], but was usually not analyzed or analyzed together with the membranous EGFR staining as a "total EGFR". It is already known that cells may produce soluble forms of EGFR (sEGFR), which contain only the extracellular ligand-binding domain but lack the ability to activate the intracellular signaling cascade [32]. Soluble EGFR particles were to date detected in the cytoplasm of normal and cancer cells and in various biological fluids [32]. Unfortunately, their exact biological function remains unclear. It was demonstrated that sEGFR present in the plasma of NSCLC patients is a positive prognostic factor [33,34], probably due to its anti-proliferative properties [35]. However, sEGFR detected in NSCLC cells differed significantly from the isoforms present in the normal tissues and plasma [32]. In our research, we noticed that cytoplasmic EGFR level in AC samples was positively associated with tumor grade. This observation allows us to assume that sEGFR expression in the cytoplasm of AC cells may play a tumor-promoting role.

It has been shown that membranous EGFR expression, although common in NSCLC, has no prognostic value [36,37]. Our results seem to confirm these findings: we had not observed any significant association between EGFR M expression and patients' survival. However, there was a difference in EGFR M expression in relation to tumor histology: mean EGFR M scores were significantly higher in LSCC compared to those in AC tumors. Our observations are in line with previous results [37–39] and support the findings that EGFR M expression was higher in LSCC than in lung tumors with non-squamous histology.

A unique feature of our study was the analysis of *EGFR* mRNA expression exclusively in tumor cells with the use of mRNA-based chromogenic *in situ* hybridization (CISH). Usually, when the gene expression is analyzed using RealTime PCR or similar methods, the tissue must be homogenized to isolate the mRNA. As a result, the analyzed sample contains a mixture of mRNA from cancer cells and other tissues, such as normal pneumocytes or respiratory epithelium. The use of the CISH method creates the possibility to analyze mRNA expression in the selected cell type, without the interference from the surrounding tissues. Post-hoc analysis of our results revealed significant associations between *EGFR* mRNA gene expression and patients' survival. Interestingly, these associations were strictly dependent on tumor histology. In AC, elevated *EGFR* expression had a positive prognostic significance, whereas in LSCC reverse relationship was observed – high *EGFR* expression was associated with reduced patients' survival. Although there are numerous studies analyzing relationships between EGFR protein level and clinical-pathological data of NSCLC patients, reports on the *EGFR* mRNA expression are rather sparse. Our analysis of the AC and LSCC datasets collected in GEPIA [40] and OncoDB [41] gene expression databases revealed no associations between *EGFR* expression and overall patients' survival. Moreover, the lack of prognostic significance of *EGFR* expression was reported also by Yan *et al.* after the analysis of four NSCLC datasets collected in Oncomine database [42]. Such contradictory results are probably caused by methodological differences between the experiments. Gene expression databases usually contain data from microarray expression studies, whereas our results were obtained using CISH and subsequent digital image analysis.

Besides *EGFR*, we also examined the *SATB1* mRNA level in NSCLC cells. Our analysis revealed that SATB1 expression both in AC samples and in the whole NSCLC cohort was significantly associated with patients' age. SATB1 is already known to be one of the factors regulating the aging

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process and cellular senescence. However, we are the first to reveal its associations with patients' age in cancer samples. SATB1 has been shown to play an anti-aging role in human and mouse neurons [43,44]. It has been demonstrated that in keratinocytes, SATB1 protects cells from senescence and that its expression can be regulated by miR-21 and miR-191 microRNAs [45,46]. In our previous study, analyzing SATB1 expression in NSCLC samples, we did not observe any relationship between SATB1 protein level and patients' age [15]. It can be hypothesized that SATB1 expression increases with patients' age, but its translation into protein is suppressed by miRNA. Furthermore, we observed a positive prognostic significance of SATB1 mRNA expression in NSCLC cells, which stands in line with the previous findings by Selinger *et al.* [17], and with our own results regarding SATB1 protein expression in NSCLC [15].

The most important finding of our study was that nuclear EGFR expression was positively associated with SATB1 level in NSCLC samples. To date, SATB1 has been shown to upregulate EGFR expression in breast cancer cells (29). In glioblastoma and colorectal cancer cell lines, *SATB1* knockdown was demonstrated to decrease EGFR levels [18,19]. However, little is known about the potential SATB1/EGFR relationships in lung cancer cells. We are the first to show a positive association between the expression of these proteins in clinical samples. Interestingly, the observed associations were strongest for the EGFR expressed in cell nuclei. That may suggest a possible regulatory loop between EGFR N and SATB1, or the role of SATB1 in EGFR nuclear translocation. It was recently demonstrated that nuclear translocation of EGFR requires Akt-mediated phosphorylation at Ser-229 [48]. It is known that SATB1, on the one hand, can be activated by Akt-mediated phosphorylation [49], and on the other, has the ability to activate the PI3K/Akt pathway [49]. It could be then hypothesized that SATB1 contributes to nuclear EGFR localization by activating the PI3K/Akt pathway. Moreover, EGFR is also one of the PI3K/Akt pathway mediators [50], therefore it could theoretically activate SATB1 and protect it from degradation [49]. Further research is needed to investigate this complex network of interdependencies.

Besides the EGFR/SATB1 expression associations, we also observed positive relationships between the EGFR level and the expression of EMT-promoting transcription factors, especially SLUG N. The role of EGFR as a factor contributing to EMT was described in numerous cancers. In liver cancer, EGFR was shown to mediate EMT through Akt/GSK-3β/SNAIL pathway activation [49]. In breast cancer cells, EGFR inhibition reversed EMT by SNAIL and Twist1 downregulation [51]. In salivary adenoid cystic carcinoma, EGFR was shown to contribute to EMT by SNAIL and SLUG stabilization [52]. Moreover, EGFR is a known stimulator of SLUG-mediated reepithelialization during wound healing [52]. It was also observed that EMT is one of the mechanisms contributing to acquired EGFR-TKI resistance, but the exact mechanisms remain unknown [53,54]. However, to the best of our knowledge, we are the first to observe the positive associations between EGFR and SLUG, SNAIL, and Twist1 expression in NSCLC clinical samples. These findings support the role of EGFR as a promoter of EMT in cancer cells.

5. Conclusions

In our work, we demonstrated that EGFR expression in non-small cell lung cancer cells was positively associated both with SATB1 levels and the expression levels of the EMT-promoting transcription factors. Moreover, we established the role of SATB1 as a positive prognostic factor in NSCLC. We also for the first time analyzed *EGFR* mRNA expression exclusively in NSCLC cancer cells without the interference caused by respiratory epithelium and tumor stroma. Our analysis revealed that the prognostic significance of *EGFR* mRNA expression was dependent on tumor histology and differed significantly between AC and LSCC samples.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1. Nuclear EGFR expression and clinical-pathological data of NSCLC patients. Table S2. Cytoplasmic EGFR expression and clinical-pathological data of NSCLC patients. Table S3. Membranous EGFR expression and clinical-pathological data of NSCLC patients. Table S4. EGFR mRNA expression and clinical-pathological data of NSCLC patients. Table S5. SATB1 mRNA expression and clinical-pathological data of NSCLC patients.

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Informed Consent Statement: The study was conducted on archival clinical material collected during planned surgical procedures. Patient consent to participate was not required. The manuscript does not contain any personal information about identifiable living patients, therefore patient consent for publication was not necessary.

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