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TDP1 and TOP1 as targets in anticancer treatment of NSCLC: Activity and protein level in normal and tumor tissue from 150 NSCLC patients correlated to clinical data

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ABSTRACT

Objectives: Topoisomerase 1 (TOP1) is a drug target used in anticancer treatment of various cancer types. The effect of the TOP1 drugs can be counteracted by the enzymatic activity of tyrosyl-DNA phosphodiesterase 1 (TDP1). Thus, to elucidate the relevance of combining TDP1 and TOP1 as drug targets for anticancer treatment in NSCLC, TDP1 and TOP1 was for the first time quantified in a large cohort of paired normal and tumor tissue from NSCLC patients, and data were correlated between the two enzymes and to clinical data.

Materials and methods: TDP1 and TOP1 activity and protein concentration were measured in paired normal and tumor tissue from 150 NSCLC patients using TDP1 and TOP1 specific biosensors and ELISA. TDP1 and TOP1 activity and protein concentration were correlated to clinical data.

Results: TDP1 and TOP1 activity and protein concentration were significantly upregulated from normal to tumor tissue for the individual patients, but did not correlate to any of the clinical data. TDP1 and TOP1 activity were upregulated in 89.3% and 82.7% of the patients, respectively, and correlated in both normal and tumor tissue. The same tendency was observed for protein concentration with an upregulation of TDP1 and TOP1 in 73.0% and 84.4% of the patients, respectively. The activity and protein concentration correlated in normal and tumor tissue for both TDP1 and TOP1.

Conclusion: The upregulations of TDP1 and TOP1 from normal to tumor tissue combined with the observation that TDP1 and TOP1 did not correlate to any of the clinical data indicate that both proteins are important for development or maintenance of the tumor cells in NSCLC. Correlations between TDP1 and TOP1 indicate a biological dependency and potential co-regulation of the enzymes. These observations is encouraging in relation to using TOP1 and TDP1 as targets in anticancer treatment of NSCLC.

1. Introduction

Lung cancer accounts for most cancer related deaths worldwide among both men and women [1]. The two main types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for approx. 85% of lung cancer cases and can be further subdivided into the subtypes; adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [2,3]. The treatment of NSCLC primarily rely on surgery, radiation therapy, and chemotherapeutic treatment for the early stages and chemotherapy, immunotherapy, and targeted therapy for advanced stages of disease [2,4]. However, the survival rate among patients diagnosed with advanced NSCLC are low, indicating a requirement for additional treatment options [2,4]. Irinotecan and topotecan have shown promising results in the treatment of advanced NSCLC [5,6]. Additionally, they have the potential of initial pre-screening of treatment response since both drugs specifically inhibit the DNA-interacting enzyme topoisomerase 1 (TOP1) [7,8].

TOP1 is an essential enzyme important for the release of topological stress arising as a consequence of natural processes such as replication and transcription [9,10]. TOP1 can be trapped on DNA due to

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endogenous or exogenous DNA damages, resulting in the formation of TOP1 cleavage complexes (TOP1cc) [11]. If left unrepaired, TOP1cc will develop into cytotoxic double strand breaks (DSB) upon collision with the DNA replication forks, which can eventually lead to cell death due to accumulation of DSB [12–14]. This is exploited in the anticancer treatment of various cancer types such as ovarian cancer [15], small cell lung cancer [16], and colorectal cancer [17] by administering derivatives of camptothecin (CPT), e.g. irinotecan or topotecan, capable of introducing TOP1cc [8,18].

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is a versatile repair enzyme involved in many different repair functions due to its ability to hydrolyze 3′-phosphodiester bonds [19,20]. TDP1 is capable of removing various 3′-adducts such as phosphotyrosine, abasic sites, 3′-phosphoglycolate ends, 3′-deoxyribose phosphate ends, chain terminating agents, biotin and quenchers [21–29]. This versatility have proven challenging for the use of drugs that exert their effect by introducing 3′-adducts into the DNA as these can be repaired by the enzymatic activity of TDP1, thus counteracting the effect of the drugs. This counteracting effect have been reported for TOP1 targeting drugs such as topotecan and irinotecan [30–35], for the anticancer drugs sapacitabine [28], and for abacavir used for treatment of HIV infections [29]. TDP1 is also involved in repair of single strand breaks introduced by ionizing radiation (IR), mainly through IR-induced abortive TOP1–DNA intermediates [36]. Furthermore, studies indicate that TDP1 is involved in the repair of DSB [37] by involvement in the non-homologous end joining repair pathway [38–40]. However, removal of TOP1cc appears to be the main repair function of TDP1 [26], which is evident from studies performed in cell lines showing that loss or inactivation of TDP1 sensitize cancer cell lines to TOP1 poisons such as CPT [31,32,41] and that TDP1 overexpression can counteract the effect of TOP1 poisons [42].

The versatile repair capability of TDP1 and its ability to counteract the effect of anticancer drugs and IR make TDP1 and interesting target in anticancer therapy [28,31,43]. Especially the relevance of combining TDP1 drugs with already existing TOP1 drugs in anticancer treatment is well established in cell studies [31,32,41,42]. Despite this, almost no information is available about the activity of TDP1 in cancer tissue. We have in a pilot study [44] including paired normal and tumor tissue from non-small cell lung cancer patients found TDP1 activity to be upregulated in 100% (20/20) of the tumor samples and TOP1 activity in 82% (14/17) of the tumor samples. Liu *et al.* [45] investigated TDP1 activity in nine NSCLC patients and also found an increase in all tumor samples compared to TDP1 activity in unrelated normal tissue samples.

The few studies addressing TDP1 activity in tumor tissue indicate that TDP1 is as an interesting target in anticancer treatment as the marked upregulation of TDP1 activity from normal to tumor tissue may suggest a central dependency on TDP1 activity in tumor development or maintenance. However, the current studies investigating TDP1 activity in human tissue were based on relatively few patient samples without any clinical data available. Therefore, we aimed to investigate TDP1 and TOP1 activity and protein concentration in a large cohort of 150 NSCLC patients containing paired normal and tumor tissue for all patients with clinical data available for almost all of them to elucidate the relevance of combining TOP1 and TDP1 as drug targets in anticancer treatment of NSCLC.

2. Materials and methods

2.1. Tissue

The study was performed using normal and tumor tissue from 150 patients diagnosed with NSCLC. The samples were collected at Pathology, Aarhus University Hospital, from 2007 to 2013. Tumor resection surgery was performed for all patients as treatment of their lung cancer. Clinical information about age, sex, smoking status, NSCLC subtype, T stage, survival time, the presence of metastases, and chemotherapeutic treatment were obtained from Department of Oncology, Aarhus University Hospital.

For measurement of activity and protein concentration, fresh frozen normal and tumor tissue embedded in Tissue-Tek O.C.T. compound and stored at −80 °C was used. For immunohistochemistry, FFPE tumor tissue was used. The patients included were 18 years or older at date of operation and were diagnosed with one of the following NSCLC subtypes; adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

The tissue was cut on a Microm HM 560 Cryostat and all tissue sections were stored at −80 °C until analyses were conducted. Graphical overview of the experimental setup is illustrated in Fig. 1A. For each patient, nine 10 μm tissue sections were cut and pooled for activity measurements and ELISA. Before and after cutting the tissue sections used for analysis, a 4 μm tissue section was cut an mounted on a microscopic slide. The normal lung tissue samples used in this study were removed adjacent to the tumor tissue thereby inducing a risk of tumor cells in the normal tissue. Thus, the “before” and “after” sections were hematoxylin and eosin (HE) stained and examined by an experienced pathologist. Patient material with tumor cells or abnormal morphology in the normal tissue or no appearance of tumor cells in the tumor tissue were excluded from the study.

During the study, some of the patient samples were excluded as shown in the flow diagram in Fig. 1B. Samples from five patients were excluded prior to molecular experiments due to tumor cells or abnormal morphology in the normal tissue sections. Additionally, five patients were excluded because no tumor cell were present in the tumor sections. After molecular experiments were performed on the remaining patient samples, 15 patients were exclude due to technical problems or insufficient performance of control samples in the activity assays. Three samples were excluded due to an abnormal low OD measurement in the normal samples. Finally, 122 paired normal and tumor samples were used for further data analyses.

2.2. HE staining of fresh frozen tissue samples

Tissue sections were defrosted for 10 min at room temperature before incubation in Harris hematoxylin (PAP1) for 1 min. Subsequently, tissue sections were briefly rinsed under running water, quickly submerged in the bluing reagent, Scott’s Water, and again rinsed under running water. This was followed by 15 s submerging in 0.1% Eosin Y added approximately 250 μL of 2% acetic acid. Lastly, the sections were dehydrated with 10 quick immersions in 70% ethanol, 96% ethanol (x2), and 99% ethanol (x6) and mounted cover glass was mounted using a Tissue-Tek SCA mounting machine (SAKURA).

2.3. Preparation of tissue extract and measurement of total protein

Tissue extract for activity assays and ELISA was prepared by lysing nine 10 μm tissue sections in 270 μL ice cold 1x TDP1 buffer (20 mM Tris-HCl pH 8, 100 mM KCl, 10 mM DTT, 10 mM EDTA, and 0.05% Triton X-100) with 1x PMSF, vortexed for 5 s, and shaken on a benchtop shaker for 30 min at 4 °C. Halfway through shaking, the same amount of PMSF was added again. 80 μL extract was removed for activity, OD, and Bradford measurements. To the remaining 190 μL extract used for ELISA, NaCl was added to a final concentration of 250 mM. Both extracts were spun down for 10 min at 4 °C and 14000 rpm. Supernatant from both tubes were used for further analyses.

Total protein concentration was measured using an Implen NanoPhotometer and the Quick Start™ Bradford Protein Assay (BIO RAD) as described by the manufacturer. OD_{280} measurement were correlated to Bradford measurement and they strongly correlated ($p < 0.0001$) in both normal and tumor tissue (data not shown) indicating no difference between the two measurements.

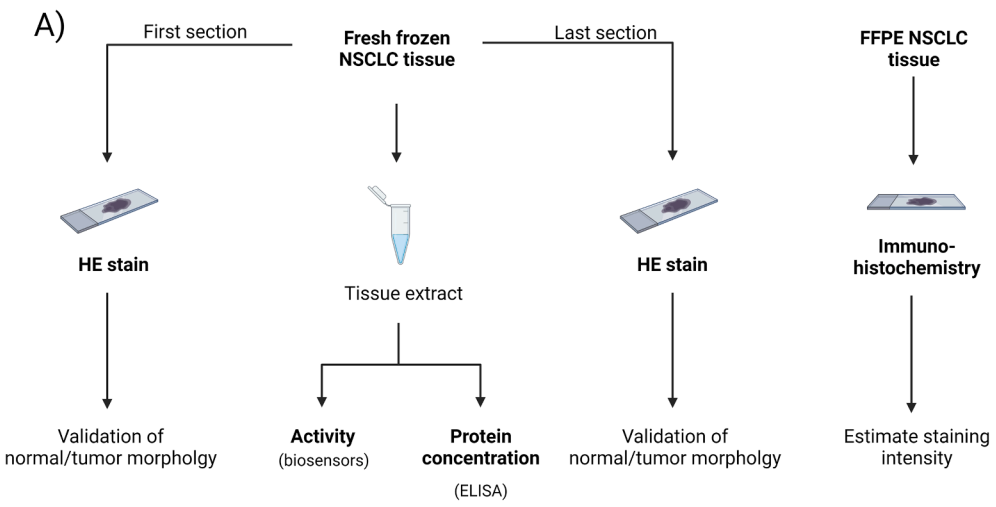

Fig. 1. Overview of study design and exclusion of patients. A) Graphical presentation of experimental setup. Fresh frozen tissue and formalin fixed and paraffin embedded (FFPE) tissue were used for the experiments. For the fresh frozen biopsies, the first and the last section were HE stained and used for microscopic evaluation of the morphology of the normal and tumor tissue. The sections between the HE stained sections were transferred to an Eppendorf tube for tissue extraction. The tissue extract was then used for measurement of TDP1 and TOP1 activity with biosensors and protein concentration with ELISA. FFPE tumor tissue was used for TDP1 and TOP1 immunohistochemistry to evaluate staining intensity in normal and tumor cells. Created with BioRender.com. B) Exclusion of patient samples. Patient samples were excluded based on abnormal morphology or technical issues. The sample number (n) represents the number of patient samples.

2.4. Synthetic DNA oligonucleotides

TDP1 biosensor: 5′-ATTO488-AAA GCA GGC TTC AAC GCA ACT GTG AAG ATC GCT TGG GTG CGT TGA AGC CTG CTT T-BHQ1-3′ (DNA technology). TOP1 substrate-Id16: 5′-AGA AAA ATT TTT AAA AAA ACT GTG AAG ATC GCT TAT TTT TTT AAA AAT TTT TCT AAG TCT TTT AGA TCC CTC AAT GCT GCT GCT GTA CTA CGA TCT AAA AGA CTT AGA-3′. Control circle with Id-33: 5′-p-AGA AAA ATT TTT AAA AAA ACT GTG AAG ATC GCT TAT TTT TTT AAA AAT TTT TCT AAG TCT TTT AGA TCC CTC AAT GCA CAT GTT TGG CTC CGA TCT AAA AGA CTT-3′. RCA primer: 5′-C6amine-CCA ACC AAC CAA CCA AAT AAG CGA TCT TCA CAG T-3′. Id16-TAMRA: 5′-TAMRA- CCT CAA TGC TGC TGC TGT ACT AC −3′. Id33-FAM: 5′-6FAM-CCT CAA TGC ACA TGT TTG GCT CC −3′.

2.5. TDP1 activity measurement

TDP1 activity was measured using a fluorescent DNA biosensor specific for TDP1 activity and described in Jensen *et al.* [27]. Immediately after preparation of tissue extract, 22.5 μL of extract was mixed with 1x TDP1-buffer and 1 μM TDP1 biosensor in a final volume of 25 μL. The negative control contained no TDP1 biosensor. Fluorescence development were measured in a black Corning 384-well plate in a FlexStation 3 Multi-Mode Microplate Reader at 494 nm excitation and 518 nm emission every 30 s for 2 h at 37 °C. Data was transferred to excel, and the linear part of the slope was used as a relative measure of TDP1 activity. All data were normalized to total protein concentration measured with an Implen NanoPhotometer. The tumor/normal activity ratio was calculated as the activity in tumor tissue divided by the activity in normal tissue.

2.6. TOP1 activity measurement

Activity of TOP1 was measured using the Rolling Circle Enhanced Enzyme Activity Detection (REEAD) assay described in Stougaard *et al.* [46]. To perform circularization of the TOP1 dumbbell substrate, 2 μL of tissue extract was mixed with 1x TDP1-buffer and 0.2 μM TOP1 substrate-Id16. The reaction was carried out for 30 min at 37 °C followed by heat-inactivation for 5 min at 95 °C. For quantification purposes, 0.05 μM ligated control circle with Id-33 was added. The rolling circle amplification (RCA) primer was coupled to CodeLink Activated Slides (SurModics) as described in the manufacturer's protocol. This was followed by addition of 5.5 μL reaction mix to the CodeLink Activated Slides and hybridization was carried out for 60 min at 37 °C in a humidity chamber. Subsequently, the slides were washed in wash buffer 1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.3% SDS) for 1 min at room temperature and wash buffer 2 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 min at room temperature. The slides were then dehydrated for 1 min in 99% ethanol and air-dried before RCA was performed. RCA mixture contained 1x Phi29 buffer, 0.3 μg/μL BSA, 250 μM dNTP, and 1 unit/μL Phi29 DNA polymerase in 3

μL and was carried out for 60 min at 37 °C in a humidity chamber. RCA reaction was stopped by washing in wash buffer 1 and 2 for 1 min each and dehydrating in 99% ethanol for 1 min. To detect the RCA products, 0.2 μM of each of the reaction probes, Id16-TAMRA and Id33-FAM, were incubated for 30 min at 37 °C with 20% formamide, 2 × SSC, and 5% glycerol in a volume of 5 μL. Finally, the slides were washed for 10 min in wash buffer 1, 5 min in wash buffer 2, dehydrated for 1 min in 99% ethanol, and mounted with Vectashield mounting medium. The fluorescent signals were visualized using a fluorescence microscope. For quantification of TOP1 activity, the number of red signals (TAMRA) and green signals (FAM) were counted in 12 pictures for each sample using the ImageJ software [47]. The relative TOP1 activity was calculated as a ratio between the number of red and green signal, followed by normalization of data to total protein concentration measured with an Implen NanoPhotometer. The tumor/normal activity ratio was calculated as the activity in tumor tissue divided by the activity in normal tissue.

2.7. TDP1 and TOP1 ELISA

TDP1 and TOP1 protein concentration were measured using sandwich ELISA kits from CUSABIO and Cloud-Clone Corp., respectively. The TDP1 ELISA kit has previously been used with tissue extract in Jakobsen *et al.* [48]. 100 μL tissue extract was used, and the assays were carried out as described by the manufacturers. The absorbance was measured in a FlexStation 3 Multi-Mode Microplate Reader at 450 nm. For TDP1, all measurements were subtracted a background measurement at 540 nm before calculating the protein concentrations using the standards series provided with the assay in a dilution series of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL TDP1. For TOP1, all measurements were subtracted a background measurement using the blank control containing no TOP1. The TOP1 protein concentrations were calculated using the standards series provided with the assay in a dilution series of 4000, 2000, 1000, 500, 250, 125, 62.5, and 0 pg/mL TOP1. Data was normalized to total protein concentration measured with an Implen NanoPhotometer.

2.8. Immunohistochemistry

Immunostaining of FFPE tumor tissue was performed using the VENTANA BenchMark ULTRA automatic immunostainer. The HPA019039 anti-TOP1 antibody (Atlas Antibodies) were used in a 1:100 dilution and the sc-365674 anti-TDP1 antibody (Santa Cruz) in a 1:50 dilution. To perform heat induced epitope retrieval, citrate buffer (pH 6) was used for TOP1, and Tris-EDTA (pH 9) was used for TDP1.

2.9. Statistics

To test if data was from a Gaussian distribution, log-transformation of the data followed by a D'Agostino & Pearson normality test were performed. Data did not come from a Gaussian distribution and nonparametric tests were used for the following analyses.

Wilcoxon signed-rank test was used to compare activity or protein concentration between normal and tumor samples. Spearman rank correlation test was used to test for all test of correlations between TDP1 and TOP1 measurements. Statistical analyses were executed using Graph Pad Prism version 9 (graphpad.com).

2.10. Ethics

The study is approved by The Central Denmark Region Committee on Health Research Ethics (project number: 1–10-72-336-16) and The Danish Data Protection Agency (project number: 1-16-02-667-16).

3. Results

3.1. TDP1 and TOP1 activity in NSCLC

TDP1 has the potential to counteract various anticancer treatments through its ability to repair DNA damages introduced by several cancer drugs and IR [28,31,43]. The counteracting effect of TDP1 upon treatment with TOP1 poisons such as irinotecan and topotecan are especially well documented [30–32,41]. However, very little information is available on TDP1 and TOP1 activity in human tissue to elucidate the relevance of combining TDP1 and TOP1 as drug targets in anticancer treatment. Therefore, TDP1 and TOP1 activity were measured in paired normal and tumor tissue from a big cohort of 150 NSCLC patients. The experimental setup is depicted graphically in Fig. 1A, a flowchart of the exclusion criteria of the biopsies in Fig. 1B, and a description of the cohort in Table 1.

TDP1 and TOP1 activities in normal and tumor tissue from 122 NSCLC patients are depicted in Fig. 2A and B, respectively. From the graphs, it can be observed that TDP1 activity ($p < 0.0001$) and TOP1 activity ($p < 0.0001$) were significantly upregulated from normal to tumor tissue. TDP1 activity were upregulated from normal to tumor tissue in 109 out of 122 (89.3%) samples, and TOP1 activity in 101 out of 122 (82.7%) samples.

To examine the association between TDP1 and TOP1 activity in NSCLC, TDP1 and TOP1 activity were correlated in normal tissue (Fig. 2C) and in tumor tissue (Fig. 2D). TDP1 and TOP1 activity correlated significantly in both normal ($p < 0.0001$, $r = 0.6765$) and tumor tissue ($p < 0.0001$, $r = 0.4855$). Additionally, there was a strong correlation between the tumor/normal activity ratio for TDP1 and TOP1 (Fig. 2E, $p < 0.0001$, $r = 0.4795$).

Age, sex, smoking status, NSCLC subtype, T stage, survival time, the presence of metastases, and chemotherapeutic treatment were correlated to tumor/normal activity ratio for TDP1 and TOP1 but no correlations was found to any of the included clinical parameters.

3.2. TDP1 and TOP1 protein concentration in NSCLC

To investigate for differences in TDP1 and TOP1 protein

Table 1
Presentation of the 122 NSCLC patients with TDP1 and TOP1 activity and protein data.

Characteristics		n/total	%
Age	40–49 years	3/122	2.5
	50–59 years	18/122	14.8
	60–69 years	40/122	32.8
	70–79 years	48/122	39.3
	80–89 years	13/122	10.7
Sex	Male	73/122	59.8
	Female	49/122	40.2
Smoking status	Smoker	82/97	84.5
	Non-smoker	15/97	15.5
NSLCLC subtype	Adenocarcinoma	60/122	49.2
	Squamous cell carcinoma	61/122	50.0
	Large cell carcinoma	1/122	0.8
T stage	1a	10/97	10.3
	1b	16/97	16.5
	2a	48/97	49.5
	2b	8/97	8.2
	3	11/97	11.3
	4	4/97	4.1
Survival	Dead	38/97	39.2
	Alive	59/97	60.8
Metastasis	Yes	5/97	5.2
	No	92/97	94.8
Chemotherapeutic treatment	Yes	16/97	16.5
	No	81/97	83.5

Clinical information regarding smoking status, T stage, survival, metastasis, and chemotherapeutic treatment was only available for 97 of the 122 patients.

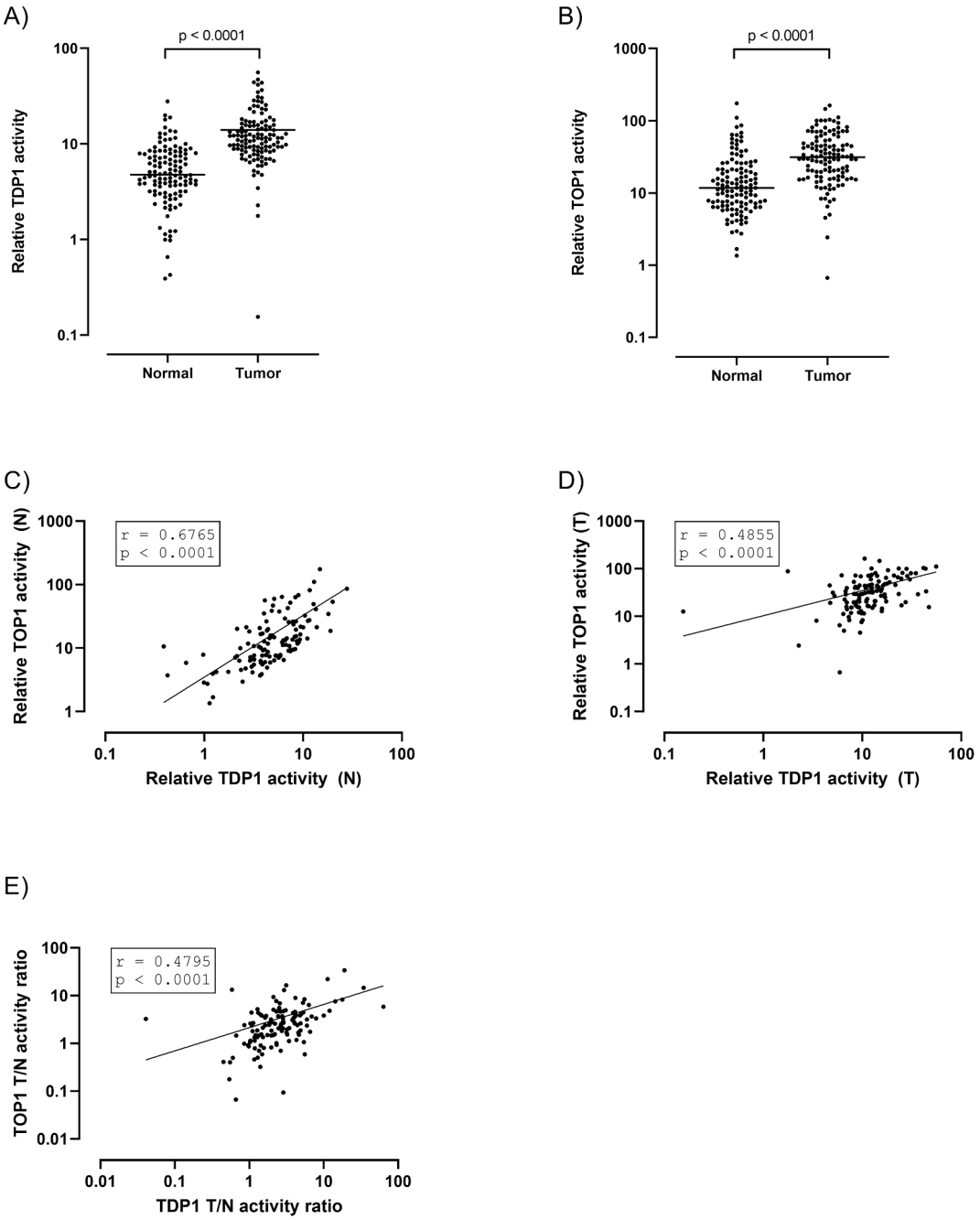

Fig. 2. TDP1 and TOP1 activity in normal and tumor tissue from 122 NSCLC patients. A) Comparison of TDP1 activity in normal and tumor tissue. The line represents the median of the activity measurements. B) Comparison of TOP1 activity in normal and tumor tissue. The line represents the median of the activity measurements. C) Correlation of TDP1 and TOP1 activity in the normal samples. D) Correlation of TDP1 and TOP1 activity in the tumor samples. E) Correlation of the tumor/normal activity ratio for TDP1 and TOP1. The tumor/normal activity ratio was calculated as activity in tumor samples divided by activity in normal samples (T/N). T = tumor tissue, N = normal tissue.

concentrations between normal and tumor tissue in the cohort of 122 NSCLC patients, protein concentration of both proteins was measured using commercially available sandwich ELISA kits. In a previous study, we found ELISA to be a useful method for measuring protein concentration in small NSCLC biopsies as used in this study [48]. From Fig. 3, it is evident that protein concentrations were significantly upregulated from normal to tumor tissue for both TDP1 (Fig. 3A, $p < 0.0001$) and TOP1 (Fig. 3B, $p < 0.0001$). TDP1 protein concentration were upregulated from normal to tumor tissue in 89 out of 122 (73.0%) samples and TOP1 protein concentration in 103 out of 122 (84.4%) samples. Additionally, TDP1 and TOP1 protein concentration correlated in the normal tissue (Fig. 3C, $p < 0.0001$, $r = 0.7690$) as well as in the tumor tissue (Fig. 3D, $p < 0.0001$, $r = 0.5473$). Furthermore, the tumor/normal protein ratios for TDP1 and TOP1 correlated significantly (Fig. 3E, $p < 0.0001$, $r = 0.6560$).

Age, sex, smoking status, NSCLC subtype, T stage, survival time, the presence of metastases, and chemotherapeutic treatment were correlated to tumor/normal protein ratio for TDP1 and TOP1 but no correlations was found to any of the included clinical parameters.

To evaluate if protein concentration may be used as a surrogate marker of activity in cases where activity measurements of TDP1 and TOP1 are impossible, the protein concentrations of TDP1 and TOP1

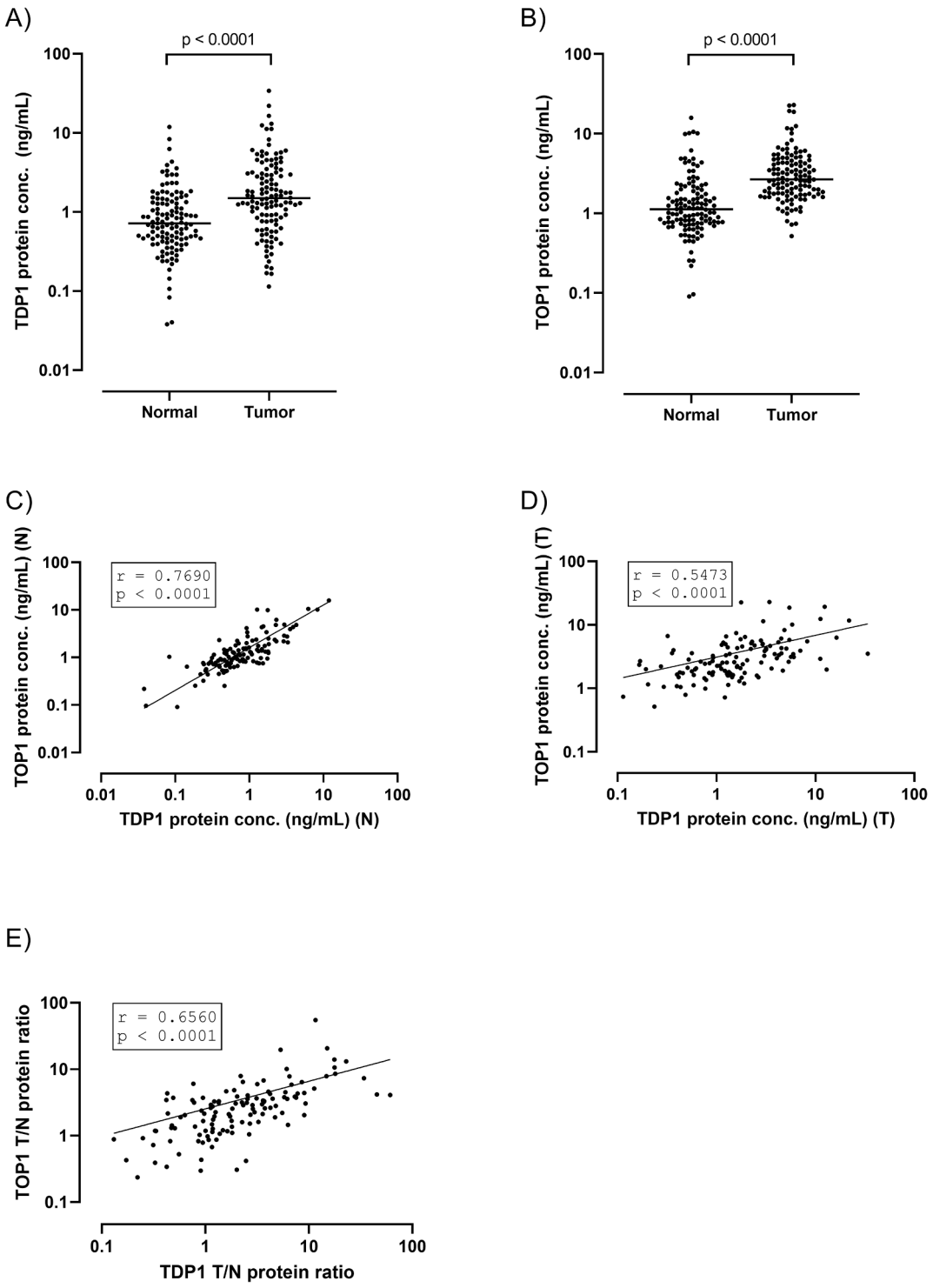

Fig. 3. TDP1 and TOP1 protein concentration in normal and tumor tissue from 122 NSCLC patients. A) Comparison of TDP1 protein concentrations in normal and tumor tissue. The line represents the median of the protein concentrations measurements. B) Comparison of TOP1 protein concentrations in normal and tumor tissue. The line represents the median of the protein concentrations measurements. C) Correlation of TDP1 and TOP1 protein concentrations in the normal samples. D) Correlation of TDP1 and TOP1 protein concentrations in the tumor samples. E) Correlation of the tumor/normal protein ratio for TDP1 and TOP1. The tumor/normal protein ratio was calculated as protein concentration in tumor samples divided by protein concentration in normal samples (T/N). T = tumor tissue, N = normal tissue.

were correlated to activity. In normal tissue, both TDP1 (Fig. 4A, $p < 0.0001$, $r = 0.4961$) and TOP1 (Fig. 4B, $p < 0.0001$, $r = 0.4348$) showed strong correlation between activity and protein concentration. The same comparison was performed in tumor tissue, where TDP1 (Fig. 4C, $p = 0.0042$, $r = 0.2573$) and TOP1 (Fig. 4D, $p < 0.0001$, $r = 0.4995$) activity and protein concentration correlated as well. Additionally, the tumor/normal activity and protein ratios correlated for both TDP1 (Fig. 4E, $p = 0.0002$, $r = 0.3285$) and TOP1 (Fig. 4F, $p < 0.0001$, $r = 0.5245$).

To determine if the observed upregulation of protein concentration were actually a result of increased TDP1 and TOP1 amount in the tumor

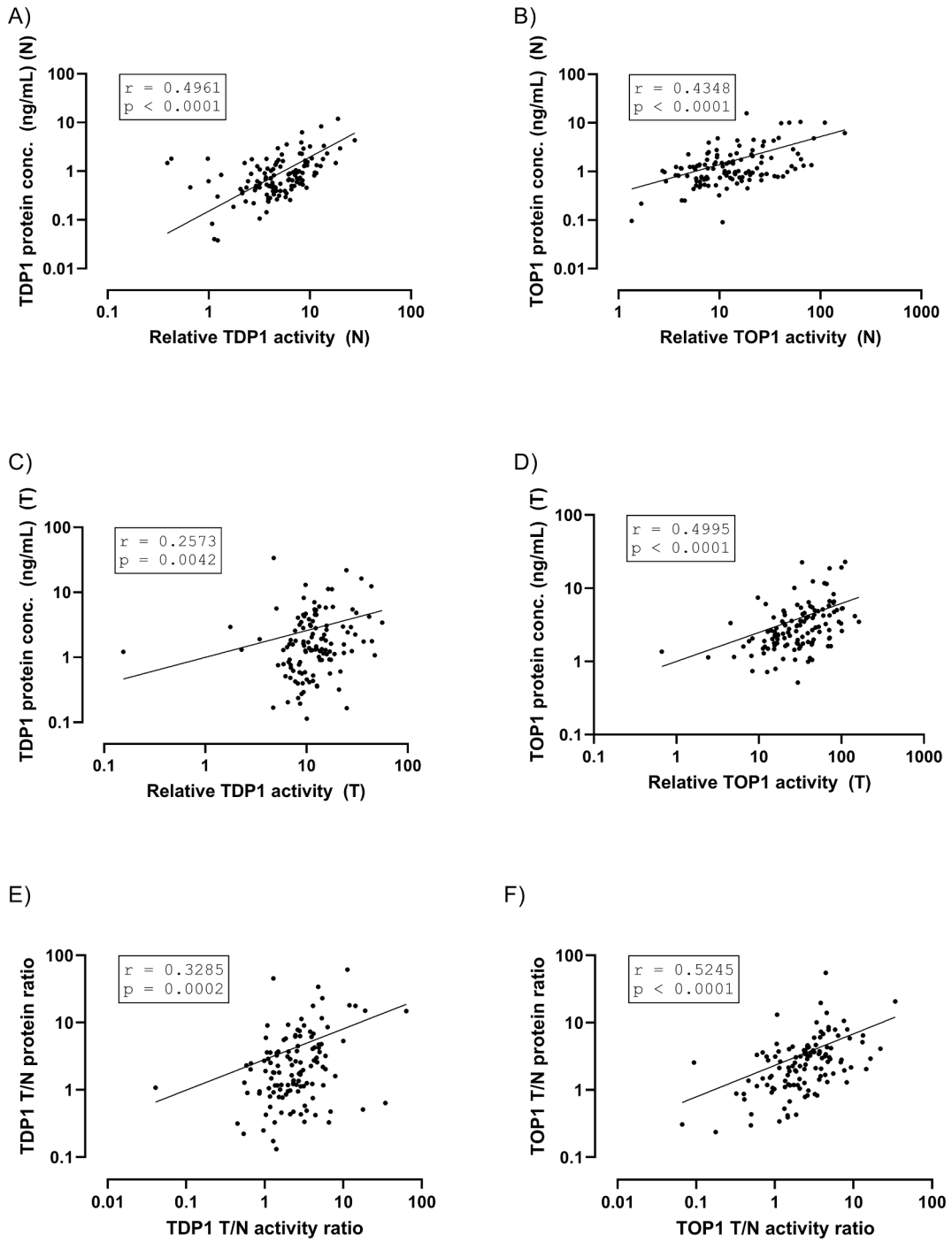

Fig. 4. Correlation of TDP1 and TOP1 activity and protein concentrations from normal and tumor tissue from 122 NSCLC patients. A) Correlation of TDP1 activity and protein concentration in normal tissue. B) Correlation of TOP1 activity and protein concentration in normal tissue. C) Correlation of TDP1 activity and protein concentration in tumor tissue. D) Correlation of TOP1 activity and protein concentration in tumor tissue. E) Correlation of the tumor/normal activity and protein ratio for TDP1. F) Correlation of the tumor/normal activity and protein ratio for TOP1. For E) and F) the tumor/normal activity and protein ratios were calculated as activity or protein concentration in tumor samples divided by activity or protein concentration in normal samples (T/N). T = tumor tissue, N = normal tissue.

cells, immunohistochemistry was performed. Immunohistochemistry can be used to visually evaluate the protein level at single cell resolution, and thereby differentiate between the cell types represented in the tissue section instead of a total protein measure achieved by quantitative methods such as ELISA and western blotting. Immunohistochemistry was performed using FFPE tumor tissue from 104 patients from the NSCLC cohort. Four TOP1 samples and eight TDP1 samples were excluded due to technical issues. TDP1 and TOP1 protein staining were identified to be primarily nuclear and in general much stronger in the tumor cell compared to the normal cells (Fig. 5A). It was observed that immune cells found in varying amounts in some tissue sections stained for both TDP1 and TOP1 (Fig. 5B). To investigate if the TDP1 and TOP1 staining in immune cells influenced the data, 40 samples were divided into three group with high, low, or no immune cells and data in the three groups were statistically compared. It was observed that the data were not different between the three groups (data not shown) and data was,

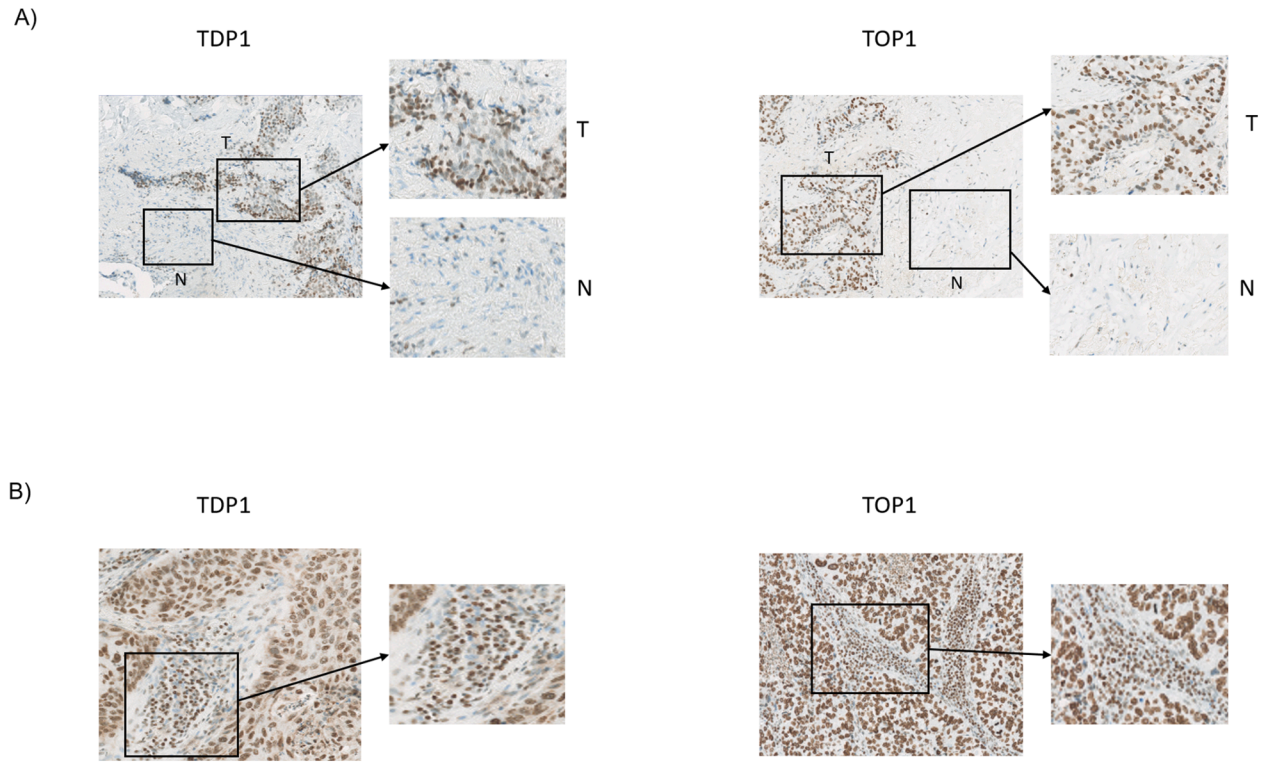

Fig. 5. TDP1 and TOP1 immunohistochemistry on FFPE tumor tissue from NSCLC patients. A) Representative pictures of TDP1 and TOP1 immunohistochemistry with staining intensity in tumor (T) cells compared to normal (N) cells. The arrows point to pictures focusing on normal cells or tumor cells. B) Representative pictures of NSCLC tumor tissue with immune cells staining for TDP1 and TOP1. The arrows point to pictures focusing on the immune cells.

thus, not adjusted in relation to immune cell content.

The potential of using mRNA level as a surrogate marker of activity for TDP1 and TOP1 were investigated as well using quantitative reverse transcription PCR. However, no correlations were found between mRNA level and tumor/normal activity or protein ratio for either TDP1 or TOP1 (data not shown).

4. Discussion

In the current study, we have for the first time in a big cohort of lung cancer patients examined the enzymatic activities and protein levels of TOP1 and TDP1. We have correlated the data to clinical parameters of the patients and examined for associations. We found a significant upregulation of TDP1 and TOP1 activity (Fig. 2A and B) and TDP1 and TOP1 protein concentration (Fig. 3A and B) from normal to tumor tissue. The upregulations were found to be caused by an upregulation in the tumor cells and not the normal cells by visually investigating the tumor tissue on a single cell resolution using immunohistochemistry (Fig. 5). The upregulations were seen in the vast majority of the tumor samples and was not associated with age, sex, smoking status, NSCLC subtype, T stage, survival time, the presence of metastases, and chemotherapeutic treatment. Thus, the upregulation of TDP1 and TOP1 seems to be independent of clinical parameters. Since all patients in the cohort were operable and, thus, in an early stage of disease, we cannot exclude that an association can be found in advanced stages of NSCLC. However, it was not possible to perform this study in advanced stages of disease since they are non-operable and normal tissue is not available.

The observation that upregulation of TDP1 and TOP1 was not associated with any of the clinical parameters indicates that TDP1 and TOP1 are likely important for development or maintenance of the tumor cells in NSCLC. This does make sense in a biological perspective where TOP1 function is important in the development of many cancer types since high cell proliferation and increased replication results in a high demand for TOP1. Increased TOP1 activity will lead to a higher requirement for repair of TOP1cc in the tumor cells. TDP1 upregulation might therefore be a direct consequence of TOP1 upregulations since TDP1-mediated repair seems to be the primary repair pathway for repair of TOP1cc [31,32,41]. This is supported by our data showing that TDP1 and TOP1 correlate on both activity level and protein level (Figs. 2 and 3). This could be a consequence of a controlled feedback mechanism between TOP1 and TDP1 expression, or TDP1 and TOP1 expression might be controlled by the same transcription factor. Such possible dependency on TDP1 and TOP1 in development or maintenance of NSCLC seems encouraging when considering TDP1 and TOP1 as targets in anticancer treatment of NSCLC.

TOP1 is already used as a target in the treatment of different cancer types such as ovarian cancer, small cell lung cancer, and colorectal cancer [15–17]. The increased TOP1 activity in the tumor tissue compared to the corresponding normal tissue in the NSCLC cohort could indicate that the use of TOP1 drugs is a promising treatment option in NSCLC. However, the studies performed so far using TOP1 targeting drugs (irinotecan and topotecan) in NSCLC have only had limited success [5,6]. Although little is known about the exact mechanisms of resistance to TOP1 drugs in NSCLC patients, several mechanisms such as efflux pumps and drug inhibiting proteins have been associated with decreased response as reviewed in Jiang *et al.* [49]. In addition, Liu *et al.* found expression of the repair protein XPF to be upregulated in NSCLC samples, thus indicating that this protein might be involved in resistance to TOP1 drugs in NSCLC [45]. Our results suggest that upregulation of TDP1 activity could be a contributing factor to the decreased response to irinotecan and topotecan in NSCLC as we found TDP1 activity to be upregulated from normal to tumor tissue in 109/122 (89.3%) of the NSCLC patients in this study. We have recently published three new TDP1 drugs and shown that they act synergistically with TOP1 drugs even at low dose. Further, we showed that the TDP1 drugs in cell line experiments had low toxicity even well above the concentration needed for a synergistic response [50]. Thus, combined targeting of TOP1 and TDP1 might be superior in NSCLC where both TOP1 and TDP1 activity is

upregulated, and may also reduce side effects as it might allow the use of reduced doses of irinotecan and topotecan.

As the activity of both TDP1 [51] and TOP1 [52–54] have been shown to be influenced by post-translational modifications, the optimal predictive value is likely measuring the activity of both enzymes. However, this requires non-fixed tissue that are not always available in clinical settings where the biopsies regularly are fixed in formalin and embedded in paraffin to preserve morphology. Thus, TDP1 and TOP1 activity and protein concentrations were correlated to investigate if protein concentration can be used as a surrogate marker of activity. Both TDP1 and TOP1 activity and protein concentration correlated in normal and in tumor tissue (Fig. 4). However, the r-values of the correlations were low (Fig. 4), indicating that the correlation of activity and protein is merely a non-perfect fit.

We here show that TDP1 and TOP1 activity are upregulated from normal to tumor tissue in more than 80% of the 122 NSCLC patients. TDP1 and TOP1 activities correlated in normal and tumor tissue and between the TDP1 and TOP1 tumor/normal activity ratios for the individual patients. Moreover, the upregulations and correlations of TDP1 and TOP1 were also observed at protein level, thereby suggesting a close biological connection in the regulation of TDP1 and TOP1 expression. We suggest that the upregulation of TDP1 may be a contributing factor to the limited response to the TOP1 poisons irinotecan and topotecan in anticancer treatment of NSCLC. Moreover, the lack of correlation to clinical data might reflect an importance of TDP1 and TOP1 in NSCLC development or maintenance.

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CRediT authorship contribution statement

Ann-Katrine Jakobsen: Writing – original draft, Conceptualization, Investigation, Formal analysis, Visualization, Funding acquisition, Project administration. **Sakineh Yuusufi:** Investigation, Formal analysis. **Line Bille Madsen:** Investigation, Supervision. **Peter Meldgaard:** Investigation, Resources, Writing – review & editing. **Birgitta R. Knudsen:** Conceptualization, Methodology, Supervision. **Magnus Stougaard:** Conceptualization, Supervision, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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