Increased expression and activity of repair genes TDP1 and XPF in non-small cell lung cancer

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Summary Resistance to camptothecin (CPT), a topoisomerase I (Top1) inhibitor, is frequently encountered in non-small cell lung cancer (NSCLC) and CPT resistance is linked with TDP1, an enzyme capable of cleaving the covalent linkage between stabilized Top1 with DNA. The aim of this study is to evaluate the in vivo expression level of TDP1, as well as parallel repair pathway components XPF and MUS81, in primary NSCLC. We collected 30 un-matched and 4 NSCLC samples matched with normal lung tissue and 8 samples of non-neoplastic lung tissue from patients with and without lung cancer, and determined the protein expression of these three genes using Western blot and TDP1 activity by a specific enzymatic assay. Both TDP1 and XPF were overexpressed in over 50% of NSCLC tissues, with wide ranges of expression levels. MUS81 did not exhibit alteration in expression. Overexpression of TDP1 and XPF is common in NSCLC, and is therefore of interest as a possible contributor to drug resistance in NSCLC.

1. Introduction

NSCLC is commonly treated by surgery or surgery with adjuvant chemotherapy, and the unresectable disease is treated locally with radiation or radiation combined with chemotherapy. In either group, response to chemotherapy or radiotherapy varies greatly with different patients.
In general, drug resistance may be related to tumor specific alterations within targeted therapeutic pathways. For example, overexpression of β-tubulin III may induce resistance to microtubule interacting drugs such as paclitaxel [1] and tamoxifen resistance is correlated with activation of PKA that causes conformational arrest of the estrogen receptor on the surface of a breast tumor [2]. For those therapies that induce DNA damage, overexpression of DNA repair genes involved in the targeted pathway may result in drug resistance. Overexpression of ERCC1, a gene involved in nucleotide excision repair (NER), is related with lower response to cisplatin therapy [3]. Topoisomerase I (Top1) inhibitor, camptothecin (CPT) analogues such as irinotecan and topotecan are commonly used in combination with platinum based compounds for NSCLC [4,5]. Drug resistance is frequently encountered, and studies on the underlying mechanisms have been carried out focusing on the repair pathways of Top1-mediated damage, and mounting evidence has pointed to the ability of Top1 to induce drug resistance in experimental systems [6–8].

Top1, a member of the phospholipase D family, is the only known enzyme capable of cleaving the phosphodiester bond between a tyrosyl residue of Top1 and the 3' phosphate of DNA, which is formed when Top1 is stabilized by CPT [9–11]. Top1 achieves this hydrolysis via attacking on the phosphate moiety of the substrate using its conserved histidine, releasing tyrosine and forming a phosphohistidine intermediate, which is further hydrolyzed, yielding a 3' phosphate and the native enzyme [9,12–14]. Some researchers have found that overexpression of Top1 in human cells caused significant reduction of DNA damage induced by CPT [6,8], while a recent study demonstrated that a point mutation of Top1 at its active site in SCAC1 patients caused CPT hypersensitivity [7]. These data suggest that Top1 might be a factor in CPT resistance, and that pharmacologic inhibition of Top1 may be useful in combination with CPT based therapy. However, the status of in vivo Top1 expression and activity in normal tissue and primary tumors is unknown. Examination of the status of the Top1-mediated repair pathway in NSCLC tissues may provide an indication of which Top1 repair pathway elements may contribute to drug resistance. Other elements in the Top1 repair pathway include, XPF, which is involved in NER by forming a complex with ERCC1 to excise the damaged DNA strand 5' from the DNA lesion [15], and MUS81. MUS81 is homologous to XPF, cleaves 3' trapped DNA in a similar way, and can resolve Holliday junctions [16,17]. XPF and MUS81 can be assumed to function in parallel to Top1 in repairing human Top1 damage based on studies of their Saccharomyces cerevisiae homologs RAD1 and MUS81 [18,36]. For XPF, although its relationship with drug resistance may be implied from the report about its partner ERCC1, its expression, as well as the expression of MUS81, has never been concordantly observed together with the expression of Top1 in NSCLC. Knowledge of the status of these three genes may help to understand which pathway may contribute to drug resistance in Top1 inhibitors therapy, and may be candidates for combined therapy.

In this study, we collected a total of 34 matched and unmatched NSCLC tissues, and observed the expression and activity of Top1. We found that the expression of Top1 had increased in more than 50% of cancer tissues, and the activity of Top1 had increased accordingly. We also observed the expression of XPF and MUS81 in these samples, and found that XPF expression had also increased in more than 50% cancer tissues and the overexpression of Top1 and XPF did not occur simultaneously in the same patients. MUS81 expression level was not found significantly altered.

2. Materials and methods

2.1. Patients

Thirty NSCLC tissues, eight non-neoplastic lung tissues including five from margins of tumors, and four pairs of matched NSCLC tissues and normal lung tissues were collected from patients at the Department of Surgery, School of Medicine, the Johns Hopkins University, after appropriate approval was obtained from the Johns Hopkins institutional review board. These tissues were snap frozen immediately after resection. Appropriate clinical information was abstracted via chart review according to previously approved protocol.

2.2. Human tissue extract

About 50–100 mg of above mentioned frozen tissues were ground in lysis buffer [150 mM NaCl, 1 mM KH2PO4, pH 6.4, 5 mM MgCl2, 1 mM EGTA, supplemented with Complete™ protease inhibitor cocktail tablets (Roche, Indianapolis, IN) in cold mortar. The homogenized mixtures were further supplemented with 0.4 M NaCl (final concentration) and incubated at 4°C for 20 min before centrifugation. The supernatants were collected, and their protein concentrations were measured.

2.3. Western blotting

Aliquots (32 μg each) of tissue extracts were loaded onto 10% acrylamide gels. After each electrophoresis and trans-
fer, TDP1, XPF and MUS81 were blotted, respectively, followed by detection of β-actin to confirm protein loading. The antibodies used were 1:1000-fold dilution of rabbit anti-TDP1 (Abcam Inc., Cambridge, MA), 1:500-fold dilution of mouse anti-XPF (clone 218) ( Trevigen, Gaithersberg, MD), 1:1000-fold dilution of mouse anti-MUS81 (Immunoquest Ltd., Cleveland, UK) and 1:3000-fold dilution of mouse monoclonal anti-β-actin (Sigma, Saint Louis, Mi). The blots were visualized using peroxidase substrate system (ECL Western blotting detection reagents, Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, UK).

2.4. TDP1 enzymatic assay

The activity of TDP1 as a phosphodiesterase to cleave tyrosyl residue was examined as described in Ref. [18–20]. Briefly, an 18-mer oligonucleotide that terminates in a 3’-phosphotyrosine (dH2N2TgAAGCCTGCTTY-3’, kindly provided by Dr. Howard Nash, NIMH) was 5’-labelled with γ-[^32P]-ATP using T4 polynucleotide kinase (New England Biolabs), and incubated with aliquots (4 or 0.08 μg) of tissue extracts or 9 ng of purified human Tdp1 (kindly provided by Dr. Howard Nash, NIMH) in a total of 12 μL reaction mix. The reaction contained 50 mM Tris–HCl, pH 8.0, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 40 μg/mL BSA and 5% glycerol. After 30-min incubation at 28 °C, reactions were stopped by the addition of 5 μL formamide sequencing buffer. Six microliter aliquots were electrophoresed on a 12% (acrylamide:bis-acrylamide 19:1) sequencing gel. To visualize bands, gels were exposed to HyBlot CL™ autoradiography film (Denville, Scientific Inc.) at −80 °C for 2 h. The lower bands in Figs. 1D and 3B showed the conversion of tyrosyl-DNA substrate to phosphoryl- and hydroxyl-DNA, which demonstrated the unique activity of TDP1 in releasing tyrosine.

3. Results

To determine TDP1 expression in NSCLC, we collected 30 NSCLC tissues of diverse histology and stage microdissected to greater than 60% purity and 8 non-neoplastic lung tissues including 5 tumor margins from different individuals as control (Table 1). We found that compared with the average expression level of control samples, TDP1 protein expression had increased in more than 50% of cancer samples by Western blot, with the levels ranging from 50% to over 30-fold after measurement using densitometry and normalization with β-actin (Figs. 1A, B and 2C). Of note, the increase in TDP1 was not correlated with clinical stage, histology and smoking history (Fig. 1A and Table 1). We selected nine tumor samples with adequate tissue and tested their TDP1 activity (Fig. 1B and D). In all the tumors examined, there were increased levels of conversion of tyrosyl-DNA to phosphoryl- and hydroxyl-DNA through TDP1 compared with control samples assayed with the same amount of cell extract. The increased activity of TDP1 generally correlated with level of expression. Remarkably, the samples that did not show increased level of TDP1 protein such as T1106 exhibited increased enzymatic activity compared with controls (Fig. 1C). This implied that TDP1 was not only induced but also activated in tumors.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
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<tr>
<td>Sex</td>
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<td>Female</td>
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<tr>
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</tr>
<tr>
<td>Non-smoker or unknown</td>
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*Age is reported as mean (range).*

XPF protein expression level was increased in more than 50% of 30 NSCLC samples compared with the average expression level of control samples on Western blots, but compared with that of TDP1, showed less dramatic fold changes, from around 50% to 10-fold as measured using densitometry and normalized with β-actin (Fig. 2A–C). XPF overexpression also showed no correlation with clinical stage, histology and smoking status (Fig. 2A and Table 1). We compared the fold changes of TDP1 and XPF overexpressed relative to normal tissues side by side, and found that in most samples TDP1 showed a greater extent of overexpression than XPF, and interestingly, those samples that highly expressed TDP1 did not exhibit high levels of XPF (Fig. 2C). We measured the protein expression level of MUS81 in nine tumors using Western blot, but did not find significant changes (Fig. 4A).

In order to determine whether the increased expression of TDP1 and XPF and the increased activity of TDP1 was cancer-patient specific or cancer-tissue specific, we further collected and evaluated four pairs of cancer versus normal tissues from the same NSCLC patients. The cancers were at stages T1N0, T2N0 and T1N2, respectively (Table 1). Consistent with our expectation, all tumor tissues showed increased level of TDP1 expression and activity when compared to normal (Fig. 3A and B). XPF overexpression was also found in three out of the four paired tumor samples (Fig. 3C). Therefore, the increased expression of TDP1 and XPF and the increased activity of TDP1 was indeed a specific feature of the cancer tissues rather than a patient-dependent phenomenon. XPF versus TDP1 expression status in samples no. 4286, 4293 and 4294 might again imply the inverse correlation of these two proteins, i.e., when one protein was at a higher level, the other one was expressed relatively less. Consistent with the previous observation, MUS81 did
Fig. 2  Expression of XPF was increased in un-matched NSCLC. (A) Quantitative analysis of the protein expression levels of XPF of 30 un-matched NSCLC vs. 8 non-neoplastic tissues. Normalization was done as described in Fig. 1A. (B) Western blotting of representative samples as shown in A. All samples were loaded with 32 μg of tissue extract. (C) Comparison of the fold changes of overexpressed TDP1 and XPF relative to the average expression level of non-neoplastic tissues. (A–C) N, non-neoplastic tissue; T, tumor.
Fig. 3 Increased expression and activity of TDP1 and increased expression of XPF were cancer-tissue specific. (A) Western blotting of TDP1 protein in four matched NSCLC. (B) Electrophoresis of the reaction products of TDP1 enzymatic assay of the same samples. All samples were assayed with both 80 ng and 4 μg of tissue extract. (C) Western blotting of XPF protein of the same samples. All samples in A and C were loaded with 32 μg of tissue extract. (A–C) N, non-neoplastic tissue; T, tumor.

Fig. 4 Expression of MUS81 in NSCLC. (A) Western blotting of four representative tumor samples vs. three non-neoplastic tissues. (B) Western blotting of four matched NSCLC. All samples in A and B were loaded with 32 μg of tissue extract.

not exhibit expression alteration in the paired normal and cancer tissues (Fig. 4B).

4. Discussion

Understanding the mechanism of drug resistance in the therapy of NSCLC is necessary for development of novel therapeutic strategies, and investigators have correlated upregulation of several well-recognized repair genes with drug resistance [3, 21, 22]. CPT induced DNA damage through topoisomerase I-DNA complex stabilization is a unique type of DNA damage, and resistance to CPT therapy has been linked with TDP1 activity [6–8]. Our report is the first one that demonstrates expression and activity of TDP1 in
primary NSCLC. We have shown that TDP1 overexpression was present in a majority of tumors when compared to normal controls as well as matched lung tissue, and was unrelated to clinical variables, albeit in a relatively modestly sized cohort. Only one patient in our cohort had undergone radiation or chemotherapy prior to operation, therefore it is unlikely that TDP1 overexpression is a result of selective pressures from radiation or chemotherapeutic administration.

Our study also discovered that expression of XPF had increased in NSCLC tissues. Interestingly, although both TDP1 and XPF contribute to the repair of TopI-mediated damage, TDP1 showed a greater extent of fold change of overexpression, and the overexpression of these two genes seemed inversely correlated. This observation may support the conclusion of a study of *S. cerevisiae* [18] that these two genes function independently of each other in repair of Top1 damage, since a coordinate correlation might be seen if they were in the same pathway. As a specific repair enzyme for the Top1-DNA complex, TDP1 is probably the cells' first choice for such damages and therefore is more intensely upregulated in most NSCLC. However, since there are several redundant pathways for the repair of Top1 damage [18, 20], it is possible that in certain circumstances, other pathways such as XPF may dominate the repair and be upregulated more even in the presence of TDP1. It is not clear why, if human *MUS81* functioned in parallel to TDP1 as it does in yeast, the gene is not correspondingly increased in primary lung cancer. However, this might be explained if MUS81 is not limiting for repair because it is present in excess of its partner subunit, Emel1. Another possibility is that MUS81 may be controlled or induced by a diverse set of regulatory elements. Further work may be required to evaluate whether XPF and MUS81 indeed function synergistically in repair of Top1 damage in humans.

It is well-recognized that DNA repair genes are stability genes or caretakers whose inactivation causes mutations in other genes to occur at a higher rate [23]. These genes are usually expected to have reduced expression and activity in cancer cells when they are involved in carcinogenesis. In our study the overexpression of TDP1 might be a physiologic compensation for increased repair demands due to faulty DNA repair or simple elevated mitotic rate. TDP1 may be particularly advantageous in that it can repair multiple forms of DNA damage [12, 24, 35, 36].

Firstly, TDP1 has been shown to be an important player in both double-strand-break repair (DSBR) and single-strand-break repair (SSBR). Top1-DNA complexes can be converted into irreversible double-strand-breaks (DSBs) when collide with replication forks [25, 26]. These complexes trap the 3' ends of DSBS, so they need to be removed before the recombination (HR) pathway for SSBR take over the repair [20, 27]. In fact, a yeast study has placed TDP1 in a RAD52-dependent SSBR pathway [20]. Single-strand-breaks (SSBS) can form when Top1-DNA complexes collide with transcription machinery or are flanked by some types of DNA lesions [28, 29]. TDP1 has been considered to play an important part in the process of SSBR [28, 30, 31] through resolving trapped Top1 residual and interacting with components of SSBR such as XRCC and DNA ligase IIIa.

In addition, TDP1's ability to remove 3' phosphoglycolates (PGs) formed by free radical-mediated oxidative damage [24, 36] gives TDP1 an important role in oxidative damage repair. Oxidative damage can be generated exogenously by tobacco, ionizing radiation or xenobiotic agents such as bleomycin, neocarzinostatin and mitomycin C, and intrinsically by enzymes of immune system as part of antimicrobial or antiviral response. Oxidative DNA damage may result in lesions such as 8-hydroxy-2'-deoxyguanosine (8-OH-dG), apurinic/pyrimidinic (AP) sites, SSBS and DSBS. An 8-OH-dG lesion can be repaired by OGG1, a well-studied repair gene whose impaired activity was found correlated with risk of lung cancer [32]. AP sites are usually repaired by endonuclease Ape1, whose polymorphisms are identified as being correlated with increased lung cancer risk [33]. The major structural alterations in SSBS and DSBS are caused by the loss of a nucleoside moiety or degradation of a sugar residual. Among the free ends, 3'PG is most refractory, and it is found that this unusual lesion can only be processed by relatively few enzymes, including TDP1 [24, 36]. In fact, the nuclear extract of cells from SCAN1 patients showed no capacity to cleave a typical 3'PG in vitro, implying that TDP1 might be the only enzyme capable of repairing this type of DNA damage. However, in spite of the importance of the enzymatic activity of TDP1, the lack of radiosensitivity of SCAN1 patients suggested that other unknown TDP1-independent pathways completed the task of 3'-PG repair [34].

TDP1 has recently reported to be able to cleave Top2-mediated damage [35]. Top2 inhibitors such as etoposide and adriamycin are frequently used in cancer chemotherapy, and if drug resistance is attributable to increased TDP1 expression and activity, the development of a TDP1 inhibitor in combination with Top2 inhibitors would be more attractive.

Finally, TDP1 has recently been shown to be able to cleave additional 3' adducts from DNA. These activities include limited DNA and RNA 3'-exonuclease activities to remove a 3' abasic site, an artificial 3'-biotin adduct from the DNA, and surprisingly, a phosphohistidine substrate accumulated by a mutant form of TDP1 with DNA [12]. Overexpression of TDP1 in cancer may imply therefore not only the resistance to CPT, but possibly a wide range of other drugs as well.

It is not clear whether expression of TDP1 and XPF are limited only in lung cancer. Further study is required to test the organ specificity of overexpression status of these genes, and determine whether measurement of expression level would help to tailor the CPT therapy of other solid tumors.

This study also provides a possible explanation at a molecular level for variation in response to CPT treatment, and implies that there is a possibility for overcoming CPT resistance by developing a TDP1 inhibitor for those patients with high levels of TDP1. In order to definitively determine the responsibility of overexpression of TDP1 for CPT resistance, we expect further experiments be done by making primary cell lines from those cancerous tissues expressing different levels of TDP1, and testing in vitro the resistance of these cell lines to CPT. If the CPT resistance is consistent with the expression level of TDP1, a genetically engineered TDP1 inhibitor can be used to knockdown the expression of TDP1 and observe whether those resistant cells can become sensitive. Regardless, these data provide an insight into the in vivo status of the major repair pathway of CPT induced DNA damage in NSCLC patients.
Conflict of interest statement

None declared.

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References


