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Acetylation on critical lysine residues of Apurinic/apyrimidinic endonuclease 1 (APE1) in triple negative breast cancers

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ABSTRACT

Protein acetylation plays many roles within living cells, modulating metabolism, signaling and cell response to environmental stimuli, as well as having an impact on pathological conditions, such as cancer pathogenesis and progression. The Apurinic/apyrimidinic endonuclease APE1 is a vital protein that exerts many functions in mammalian cells, acting as a pivotal enzyme in the base excision repair (BER) pathway of DNA lesions, as transcriptional modulator and being also involved in RNA metabolism. As an eclectic and abundant protein, APE1 is extensively modulated through post-translational modifications, including acetylation. Many findings have linked APE1 to cancer development and onset of chemo- and radio-resistance. Here, we focus on APE1 acetylation pattern in triple negative breast cancer (TNBC). We describe the validation and characterization of a polyclonal antibody that is specific for the acetylation on lysine 35 of the protein. Finally, we use the new antibody to analyze the APE1 acetylation pattern on a cohort of TNBC specimens, exploiting immunohistochemistry. Our findings reveal a profound deregulation of APE1 acetylation status in TNBC, opening new perspectives for future improvements on treatment and prognosis of this molecular subtype of breast carcinomas.

1. Introduction

The complexity of the proteome extends far beyond the mere number of protein-coding genes: several mechanisms that act downstream transcription and translation greatly expand the plethora of functions that a single protein-coding sequence can achieve. Among these, post-translational modifications (PTMs) of proteins modulate nearly all processes within living cells. More than 200 different PTMs have been reported: from the covalent functionalization of proteins with relatively large modifiers (e.g. ubiquitin), to the alteration of target polypeptides with tiny chemical moieties (e.g. methylation or acetylation) [1]. Since its discovery in histones 40 years ago, lysine acetylation has been investigated as an essential PTM that act modulating histones-DNA interactions and, ultimately, chromatin accessibility. In the last decades, however, the significance of protein acetylation has increasingly become broader, as many groups have pointed out the importance of this PTM for several non-histone proteins [2]. Acetylation, in fact, has profound consequences on protein functions and regulates many physiological processes such as cellular metabolism, migration and signaling, as well as pathological conditions, such as cancer occurrence and progression [2,3].

The Apurinic/apyrimidinic endonuclease/redox effector factor-1 (APE1/Ref-1, hereafter APE1) is an essential and multifunctional protein, firstly discovered as central enzyme in the base excision repair (BER) pathway of DNA lesions [4,5]. APE1 acts as the main abasic endonuclease within mammalian cells, contributing to the maintenance of genomic stability against alkylative and oxidative DNA damage. As the acronym suggests, the protein has also been separately identified as a transcriptional modulator, able to activate a broad range of transcription factors through a redox-mediated mechanism. In addition to these "classical" functions, APE1 is involved in other cellular processes such as RNA metabolism and transcriptional repression through the binding of negative calcium response elements (nCaRE) sequences within gene promoters [6,7]. APE1 is an abundant protein (10^6–10^7 copies/cell) and is endowed with a relatively long half-life (approximately 8 h in HeLa cells), therefore, to cope with a rapidly changing environment, cells must rely on a fast way to modulate APE1 activities and localization.

Abbreviations: APE1, Apurinic/apyrimidinic endonuclease1; PTM, post-translational modification; nCaRE, negative calcium response elements; BER, base excision repair; TNBC, triple negative breast cancer; PARP, Poly(ADP-ribose) Polymerase; MMS, methyl-methanesulphonate.

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Hence, it is not unexpected that the protein undergoes several PTMs including phosphorylation, ubiquitination, proteolysis, S-nitrosation and acetylation [4,8]. Several acetylation sites have been found within the APE1 poorly structured N-terminal region. In particular, acetylation/deacetylation on K^6 and K^7 has been linked to the modulation of several APE1 activities, including its affinity to the nCaRE sequences, the YB-1-mediated MDR1 gene activation and also the BER function of the protein, upon genotoxic insult [9–11]. Recent evidences from our laboratory, moreover, highlight the role of the lysine cluster between K^{27} and K^{35} in the modulation of APE1 functions. The acetylation status of these residues, in fact, appears to be modulated by genotoxic stress and likely influences the accessibility of K^6 and K^7 to lysine acetyl-transferases and deacetylases, suggesting the existence of a cross-talk between different acetylation hot-spots of the protein. The modification of lysines within the basic K^{27} to K^{35} cluster, has been also linked to a modulation of APE1 endonuclease activity, subcellular localization, nucleic acids binding affinity and protein–protein interaction [12] and (Lirussi et al., unpublished results). Moreover, acetylation of the K^{27} to K^{35} cluster induced by alkylating treatment with methyl-methanesulphonate (MMS), is associated with an increased DNA repair activity of APE1 which may account for the reduced sensitivity of some cancer cells to DNA damaging agents (Lirussi et al., unpublished results). As a central enzyme in the BER pathway, APE1 has in fact been linked to tumorigenesis and onset of chemo- and radio-resistance in several malignancies. In particular, aberrant APE1 localization and expression patterns, have been related to an increased tumor aggressiveness and recurrence, lymph node positivity and poor prognosis [13–16]. However, there are no data linking the acetylation status of APE1 and tumor progression.

In this paper, we investigated the status of APE1 acetylation, on the K^{27} to K^{35} cluster, in triple negative breast cancer (TNBC). We developed and characterized a polyclonal antibody specific for acetylated APE1 that we exploited to analyze, through immunohistochemistry, a cohort of TNBC specimens. Our data point to a profound deregulation of APE1 acetylation status in specific subtype of breast carcinoma and represent a descriptive starting point for future translational studies devoted to improve treatment and prognosis of this disease.

2. Materials and methods

2.1. Peptide synthesis and purification

Different APE1 peptide regions (Table 1) containing $K^{27}/K^{31}/K^{32}/K^{35}$ were chemically synthesized in the acetylated and non-acetylated form. Solid phase peptide synthesis was performed on an automated synthesizer Syro I (MultiSynTech), following Fmoc methodologies [17]. Peptides were purified by preparative RP-HPLC on a Shimadzu LC-8A, and analyzed through mass spectrometry. LC-ESI-Ion Trap (IT)-MS analysis was carried out on a Surveyor HPLC system connected to a LCQ DECA XP IT (Thermo Scientific).

Table 1
Sequences of the peptides used in this study.

Peptide name	Sequence[a]
25–38	^{25}KS**K**TAA**KK**ND**K**EAA^{38}
25–38^{27,31,32,35Ac}	^{25}KS**K**(Ac)TAA**K**(Ac)**K**(Ac)ND**K**(Ac)EAA^{38}
24–39	^{24}KKS**K**TAA**KK**ND**K**EAAG^{39}
24–39^{27,31,32,35Ac}	^{24}KKS**K**(Ac)TAA**K**(Ac)**K**(Ac)ND**K**(Ac)EAAG^{39}
14–38	^{14}GDELRTEPEAKKS**K**TAA**KK**ND**K**EAA^{38}
14–38^{27,31,32,35Ac}	^{14}GDELRTEPEAKKS**K**(Ac)TAA**K**(Ac)**K**(Ac)ND**K**(Ac)EAA^{38}
14–38^{31,32,35Ac}	^{14}GDELRTEPEAKKS**K**TAA**K**(Ac)**K**(Ac)ND**K**(Ac)EAA^{38}
14–38^{27,31,32Ac}	^{14}GDELRTEPEAKKS**K**(Ac)TAA**K**(Ac)**K**(Ac)ND**K**EAA^{38}
14–38^{35Ac}	^{14}GDELRTEPEAKKS**K**TAA**KK**ND**K**(Ac)EAA^{38}

[a] Numbers indicate the position of the peptide within APE1 amino acidic sequence. "Ac" denotes acetylated residues. $K^{27}/K^{31}/K^{32}/K^{35}$ are bolded.

2.2. Generation of polyclonal antibody specific for acetylated APE1

Based on the identified acetylation sites at $K^{27}/K^{31}/K^{32}/K^{35}$, we synthesized a peptide bearing the APE1 25–38 sequence and acetylated lysine residues, which was conjugated through the N-terminal CG dipeptide with keyhole limpet hemocyanin (KLH), i.e. KLH-CG^{25}KSK(Ac)TAAK(Ac)K(Ac)NDK(Ac)EAA^{38}. Polyclonal antibodies against this peptide were raised in rabbits following standard procedures (PRIMM).

2.3. In vitro acetylation of recombinant APE1

Recombinant full length APE1 mutants were previously described (Lirussi et al., unpublished results). Both recombinant proteins or APE1 peptides were non-enzymatically acetylated by incubation with 5 mM acetyl-CoA [18] and (Lirussi et al., unpublished results), in HAT buffer (50 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 5% glycerol, 1 mM DTT) for 2 h at 30 °C. Reaction was stopped by adding Laemmli sample buffer and subjected to immunoblotting using the indicated antibodies. The acetylation of proteins and peptides was confirmed through MS analysis.

2.4. Cell culture, transfection and immuno-precipitation

Cell culture, transfection and immuno-precipitation have been performed by using already published methodologies [12].

2.5. Immuno-fluorescence and immunohistochemistry

Immuno-fluorescences with the anti-APE1$^{K27-35Ac}$ (PRIMM) have been carried out following previously described procedures [12]. Briefly, cells were paraformaldehyde-fixed for 20 min at RT and permeabilized for 5 min using a PBS, 0.5% (v/v) Triton X-100 solution. After saturation with 10% (v/v) foetal bovine serum (Euroclone) for 30 min, slides were incubated with the polyclonal anti-APE1$^{K27-35Ac}$ antibody (1:100 for 3 h at 37 °C), followed by a secondary anti-rabbit Dyt-Light549 conjugated (Jackson Immuno-Research). Microscope slides were visualized through a TCS SP laser-scanning confocal microscope (Leica).

Core biopsies of triple negative breast tumors were examined by immunohistochemistry. All tissues were formalin-fixed for 16–24 h. Five micrometers formalin-fixed paraffin-embedded tissue sections, mounted on Superfrost slides (Surgipath) were immunohistochemically stained by using the peroxidase/DAB Plus Dako REAL™ EnVision™ Detection System (Dako A/S). Antigen retrieval was performed in a water bath at 98 °C with 0.01 M citrate buffer, at pH 6.0 for 40 min. Endogenous peroxidase activity was blocked by incubation in the Peroxidase Block solution (Dako A/S) for 10 min. Primary mouse monoclonal anti-APE1 antibody [20] diluted 1:200 (optimum working dilution) and primary rabbit polyclonal anti-APE1$^{K27-35Ac}$ (PRIMM) antiserum diluted 1:100 were applied to each slide and incubated, respectively, for 60 min at room temperature and overnight at 4 °C. After washing, slides were incubated with the peroxidase/DAB Plus Dako REAL™ EnVision™ Detection System (Dako A/S) according to manufacturer's guidelines. For reaction visualization, 3-3 diaminobenzidine tetrahydrochloride was used as chromogen. The sections were counterstained with Mayer haematoxylin. Normal breast tissue sections were used as positive control, whereas negative control was performed by replacing the primary antibody with PBS. Controls were included in each staining run. Nuclear immunostaining was quantitatively evaluated by using light microscopy: the entire section was scanned at high-power magnification (400×) and the percentage of positive cells was scored.

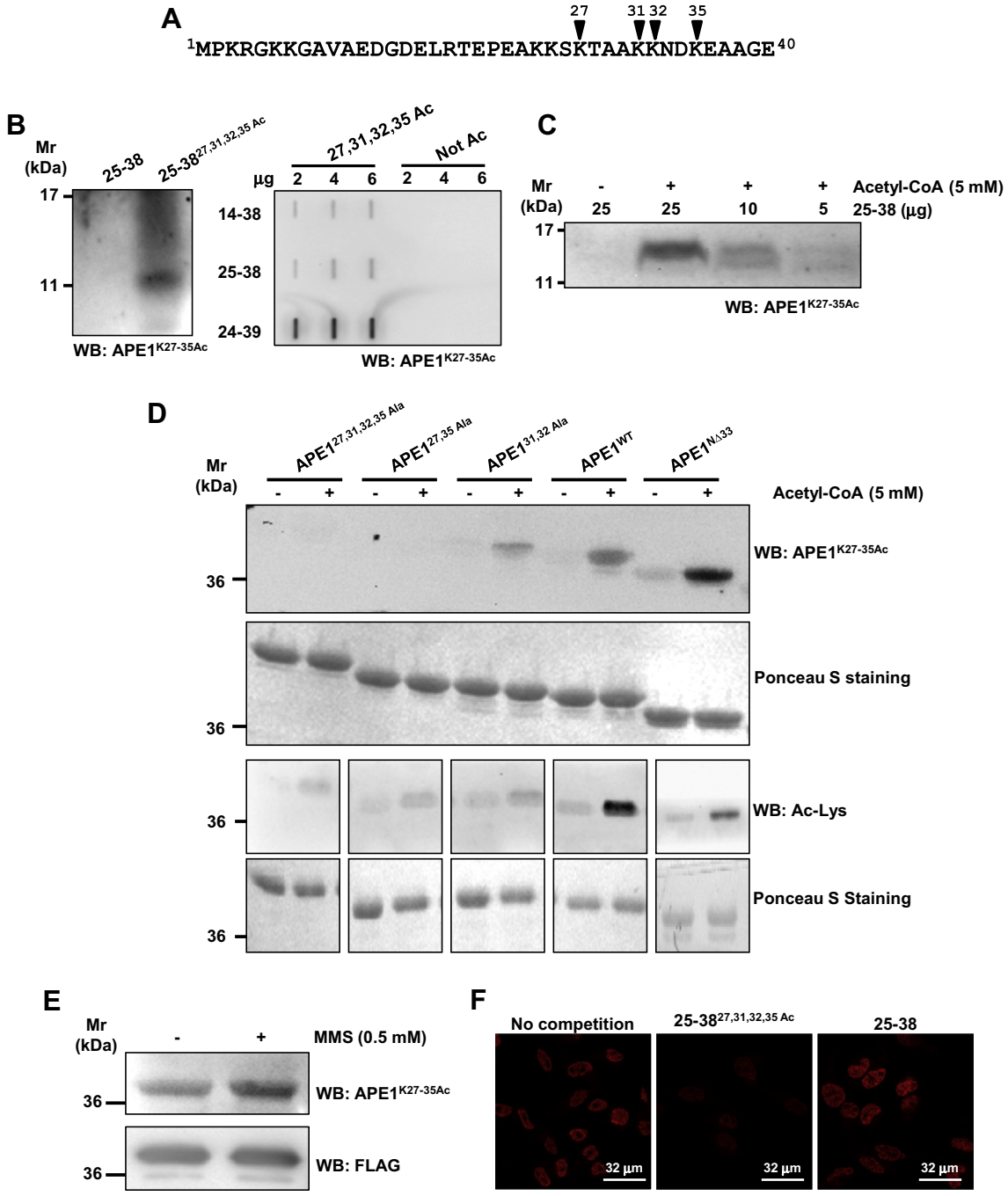

Fig. 1. The anti-APE1$^{K27-35Ac}$ antibody is specific for APE1 acetylation at $K^{27}/K^{31}/K^{32}/K^{35}$. (Panel A) Schematic view of the position of the acetyl-lysine residues in the primary sequence of APE1. Lysines residues targeted by the antibody are indicated by an arrowhead. (Panel B) Western blotting analyses on synthetic APE1 peptides. Left panel: 10 µg of chemically synthesized 25–38 or tetra-acetylated 25-38^{27,31,32,35Ac} were separated onto a discontinuous Tris-tricine SDS–PAGE and then subjected to immunoblotting. Right panel: the indicated amounts of BSA-conjugated 24–39 APE1 peptide, or unconjugated 14–38 and 25–38 APE1 peptides (either in their non-acetylated or tetra-acetylated forms) were slot-blotted on PVDF membrane and subjected to immunoblotting. (Panel C) Western blotting analysis on the *in vitro* acetylated 25–38 peptide; increasing amounts of the non-acetylated 25–38 peptide were *in vitro* acetylated as described in Section 2 and separated onto a discontinuous Tris-tricine SDS–PAGE prior to immunoblotting analysis. All Western blotting analyses were performed using the anti-APE1$^{K27-35Ac}$ antibody. (Panel D) The anti-APE1$^{K27-35Ac}$ antibody recognizes *in vitro* acetylated full length APE1. 2.5 µg of recombinant APE1 and its lysine to alanine mutants were *in vitro* acetylated as described in Section 2 and probed with either a commercial anti-Acetyl-lysine antibody (Millipore) or the anti-APE1$^{K27-35Ac}$ antibody. Ponceau-S staining was used as a loading control for normalization. (Panel E) The anti-APE1$^{K27-35Ac}$ antibody detects changes in the APE1 acetylation pattern upon MMS treatment. FLAG-APE1 was immuno-precipitated from HeLa cells treated for 4 h with MMS, as indicated, was probed using the anti-APE1$^{K27-35Ac}$ antibody. Anti-FLAG (Sigma) was used as loading control. (Panel F) Immuno-fluorescence on HeLa cells performed using either the anti-APE1$^{K27-35Ac}$ alone, or in presence of a 50-fold molar excess of 25–38^{27,31,32,35Ac} or 25–38 peptides. Images were captured maintaining fixed photomultiplier and laser parameters.

2.6. Western blotting analyses

Protein and peptides were separated onto 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) or onto a discontinuous Tris-tricine SDS–PAGE [19] and transferred to nitrocellulose or PVDF membranes (Perkin Elmer). Western blotting was carried out as already described [20], using 5% (w/v) bovine serum albumin (BSA) as blocking agent and revealed using ECL chemiluminescence procedure (GE Healthcare). Images were acquired through a ChemiDoc XRS video-densitometer (Bio-Rad).

2.7. Surface plasmon resonance (SPR)

The BIAcore 3000 SPR system for Real time binding assay and related reagents were from GE Healthcare. The polyclonal anti-APE1$^{K27-35Ac}$ antibody was immobilized at a concentration of 25 μg/mL in 10 mM acetate buffer pH 5.0 (flow rate 5 μL/min, time injection 7 min) on a CM5 Biacore sensor chip, using EDC/NHS chemistry following the manufacturer's instructions [21]. Residual reactive groups were deactivated by treatment with 1 M ethanolamine hydrochloride, pH 8.5. Reference channel was prepared by activating with EDC/NHS and deactivating with ethanolamine. Binding assays were carried out at 20 μL/min, with 4.5 min contact-time, APE1 peptides were diluted in running-buffer, HBS (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4) 0.1 mM TCEP. Analyte injections of 90 μL were performed at the indicated concentrations. The BIAevaluation analysis package (v4.1, GE Healthcare) implemented by instrument software was used to subtract the signal of the reference channel and to evaluate kinetic and thermodynamic parameters of complexes.

3. Results and discussion

3.1. Specificity of anti-APE1$^{K27-35Ac}$ antibody

To gain new insights into the role of acetylation in the functional modulation of APE1, we developed a polyclonal antibody in rabbit. The antibody (hereafter referred to as anti-APE1$^{K27-35Ac}$) was developed to recognize the tetra-acetylated form of the APE1 N-terminal 27 to 35 lysines stretch (Fig. 1A). The anti-APE1$^{K27-35Ac}$ antibody specificity was tested both by Western blotting and slot blotting, using a panel of chemically synthesized APE1 25–38 or 24–39 peptides (Table 1) that were either acetylated during synthesis (Fig. 1B) or *in vitro* acetylated after synthesis (Fig. 1C). Immunoblotting analyses confirmed the ability of the antibody to discriminate the acetylated peptides from their non-acetylated counterparts. The ability of the anti-APE1$^{K27-35Ac}$ antibody to recognise APE1 acetylation within a full-length protein background was investigated using purified recombinant lysine to alanine APE1 mutants expressed in *Escherichia coli* (i.e. APE1^{WT}, the double mutants APE1^{27,35Ala} and APE1^{31,32Ala}, the quadruple mutant APE1^{27,31,32,35Ala} and the deletion mutant APE1$^{N\Delta33}$, that lacks the entire 1 to 33 N-terminal domain) (Lirussi et al., unpublished results). Recombinant proteins were *in vitro* acetylated using acetyl-CoA and subjected to Western blotting (Fig. 1D). Analyses revealed that the anti-APE1$^{K27-35Ac}$ is able to detect APE1 acetylation only in presence of an acetylatable (i.e. not mutated) lysine residue at position 35 (Fig. 1D – upper panel). The specificity of the anti-APE1$^{K27-35Ac}$ antibody was also compared to that of a commercial anti-Acetyl-lysine antibody (Millipore); this antibody is also able to discriminate between acetylated APE1 forms, though with a different pattern. In particular, all APE1 mutants are detected by the Acetyl-lysine antibody when in their acetylated form, in accordance with the broad specificity of the antibody and with the presence of other acetylatable residues within APE1 (e.g. K^6 and K^7) (Fig. 1D – lower panel). Altogether these data suggest that the anti-APE1$^{K27-35Ac}$ specifically recognizes APE1 acetylation within the 27 to 35 lysines stretch, with a particular affinity toward the acetylated K^{35} residue. This latter point is further highlighted by the strong signal obtained with the APE1$^{N\Delta33}$ deletion mutant, which conserves only K^{35}, that is possibly located in a more accessible position. To further validate the specificity of the anti-APE1$^{K27-35Ac}$ antibody, we used immuno-precipitated APE1 samples obtained from HeLa cells treated with MMS, to increase the acetylation status of the K^{27} to K^{35} cluster (Lirussi et al., unpublished results). Fig. 1E shows that the anti-APE1$^{K27-35Ac}$ efficiently detects changes in the acetylation pattern of the protein. We finally assessed the staining pattern of the antibody using immuno-fluorescence on HeLa cells: the selectivity of the staining was verified through competition studies using the acetylated 25–38 peptide (Fig. 1F).

Surface Plasmon resonance (SPR) biosensors are ideally suited for antibody characterization and quantitative immunoassay detection. Here, we exploited SPR to measure the affinity of the antibody for the acetyl-lysine residues within APE1 amino acids 27 to 35 (Fig. 2). To characterize the kinetic values for the anti-APE1$^{K27-35Ac}$ we employed different APE1 peptides as ligands (Table 1). SPR data confirmed what observed through Western blotting, showing that acetylated K^{35} is the preferential target site for immunorecognition, with a measured K_D in the sub-micromolar range (Table 2). Notably, the lack of acetylation on K^{35} only, results in a dramatic reduction in the antibody affinity for the cognate peptide (Fig. 2B, Table 2 and data not shown). Therefore, these analyses lead us to conclude that the developed antibody has major specificity toward the acetylated K^{35} residue of APE1.

3.2. Analysis of APE1 acetylation in triple negative breast Cancer

The APE1$^{K27-35Ac}$ antibody was used to study APE1 acetylation in a cohort of triple negative breast cancer (TNBC) specimens. This type of breast cancer comprises tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR) and overexpression/gene amplification of epidermal growth factor receptor type 2 (HER2) [22]. TNBC account for approximately 15% of all invasive breast cancers and often show aggressive phenotypical characteristics, such as a high histologic grade [23,24]. To date, no specific molecular targets have been identified in TNBC and, accordingly, no specific targeted therapies have been developed [25]. Therefore, the prognosis of patients with TNBC is usually poorer than that of

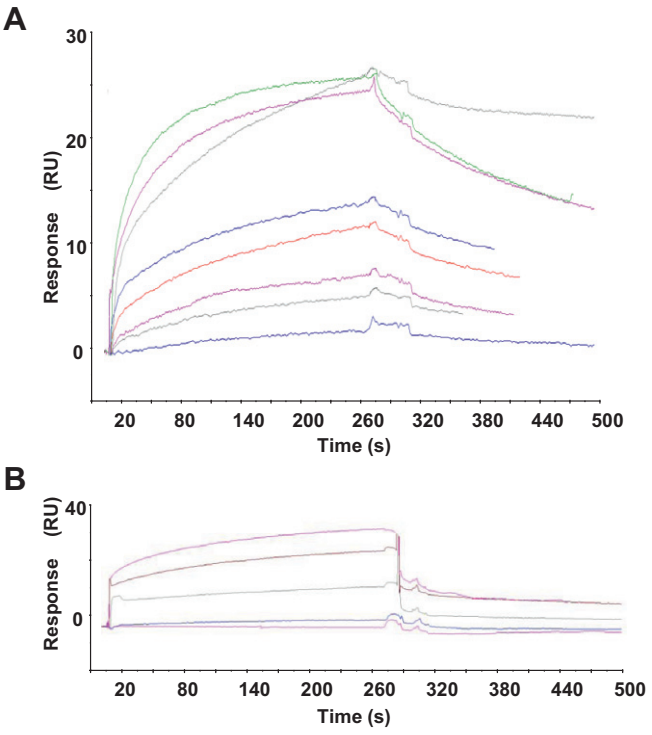

Fig. 2. The anti-APE1$^{K27-35Ac}$ antibody displays a major specificity for the acetylated lysine **35.** (Panel A) Representative overlay sensorgrams relative to a typical SPR experiment of immobilized anti-APE1$^{K27-35Ac}$ at a 2000RU level with either A the APE1 14–38^{27,31,32,35Ac} (5–800 nM) peptide or B the APE1 14-38^{27,31,32Ac} (0.1–1000 μM) peptide.

Table 2
Kinetic and thermodynamic parameters of anti-APE1$^{K27-35Ac}$ toward peptides described in Table 1.

Peptide	$K_a \times 10^4$ (Ms)	$K_d \times 10^{-3}$ (1/s)	K_D (M)
14–38	ND[a]	ND	$\sim 10^{-3}$
14–38^{35Ac}	2.39	6.52	$270 \pm 8 \times 10^{-9}$
14–38^{31,32,35Ac}	4.70	7.71	$164 \pm 3 \times 10^{-9}$
14–38^{27,31,32Ac}	ND	ND	$\sim 10^{-3}$
14–38^{27,31,32,35Ac}	3.62	5.10	$141 \pm 3 \times 10^{-9}$

[a] ND: Not Determined.

patients with other subtypes of breast cancer [26,27]. Various findings, however, indicate that a substantial diversity in biology, prognosis and treatment sensitivity can be observed among tumor defined as TNBC [28,29]. A cohort of 103 TNBC was investigated for levels of total APE1 and acetylated APE1 by using immunohistochemistry. Fig. 3 shows representative immunostainings and quantitation of positivity: overexpression of total APE1 was detected in TNBC compared to normal breast tissue, with 90% of tumor specimens showing an APE1 nuclear positivity higher than 80% (Fig. 3B). This result is in accordance with previous works that report a correlation between APE1 overexpression and tumor aggressiveness in a variety of cancers [4,6]. In contrast, immunostaining of acetylated APE1 was very heterogeneous among TNBC specimens: samples showing either low or high nuclear positivity were observed (T1 and T2, respectively, in Fig. 3A). The heterogeneity of APE1$^{K27-35Ac}$ immunostaining among TNBC is quantitatively captured in Fig. 3B. These data suggest that in TNBC, concomitantly with total APE1 overexpression, a specific deregulation of the acetylation status of this protein is occurring. APE1 deregulation in breast carcinoma (present data and [30]) may be linked to an impairment in its redox activity, being instrumental in altering estrogen-responsive gene expression in breast cancer cell lines [31].

Member of the Poly(ADP-ribose) Polymerase (PARP) family are central to specific DNA damage repair pathways, particularly the BER. PARP inhibition has been shown to enhance the cytotoxicity of agents that generate single-strand breaks in DNA, such as radiation and certain chemotherapy drugs (i.e. alkylating agents) [32]. In addition, PARP inhibitors may induce cell death through "synthetic lethality", if the DNA repair mechanisms that rescue BER-deficient cells are themselves impaired, as in BRCA-deficient breast cancers [32,33]. Because of the phenotypic similarities between some TNBC and most BRCA1-associated breast cancers, some have hypothesized that TNBC might also be sensitive to PARP inhibition [32]. Interestingly, it has been recently demonstrated that BRCA1 may be involved in regulation of the expression levels of DNA repair genes such as APE1, OGG1 and NTH1 [34]. Thus, APE1 represents an ideal candidate target for developing new therapeutic strategies for breast cancer treatment. In particular, deregulation of APE1 observed in breast cancer specimens might be exploited in order to induce synthetic lethality in tumors with defects in other DNA repair pathway, as recently pointed out [33]. Our observations, therefore, represent a descriptive starting point for future investigations; it would be interesting to understand

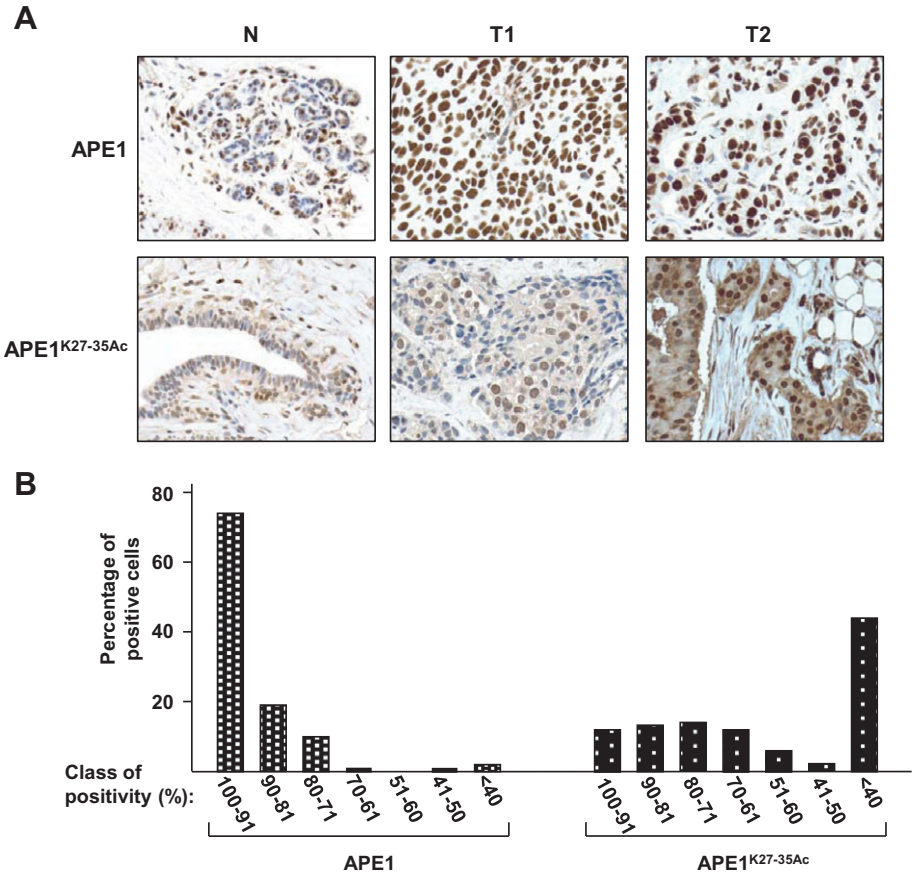

Fig. 3. Immunostaining of TNBC specimens for total APE1 and acetylated APE1. (Panel A) Representative cases of normal breast tissue (N) and TNBC (T1 and T2) stained with antibody against total (APE1) and acetylated (APE1$^{K27-35Ac}$) APE1. Positivity is indicated by the brown colour. (Panel B) Quantitative analysis of nuclear positivity for total APE1 and acetylated APE1. In x and y axes are indicated different classes of positivity and percentage of TNBC present within each class, respectively. APE1 or APE1$^{K27-35Ac}$ immunostaining is indicated below the respecting bars.

whether the deregulation of APE1 acetylation status is paralleled by an impairment of its functions, in order to design novel therapeutic approaches to breast carcinoma.

Conflict of Interest

Authors declare that they have no conflict of interest pending.

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