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Elevated APE1 dysregulates homologous recombination and cell cycle driving genomic evolution, tumorigenesis and chemoresistance in esophageal adenocarcinoma

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Abstract

Background and Aims: Purpose of this study was to identify drivers of genomic evolution in esophageal adenocarcinoma (EAC) and other solid tumors.

Methods: An integrated genomics strategy was used to identify deoxyribonucleases correlating with genomic instability (as assessed from total copy number events in each patient) in six cancers. Apurinic/apyrimidinic nuclease 1 (APE1), identified as top gene in functional screens, was either suppressed in cancer cell lines or overexpressed in normal esophageal cells, and impact on genome stability and growth monitored in vitro and in vivo. Impact on DNA/chromosomal instability was monitored using multiple approaches including investigation of micronuclei, acquisition of single nucleotide polymorphisms, whole genome sequencing (WGS) and/or multicolor fluorescence in situ hybridization.

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*SK, JZ and ST contributed equally to this work

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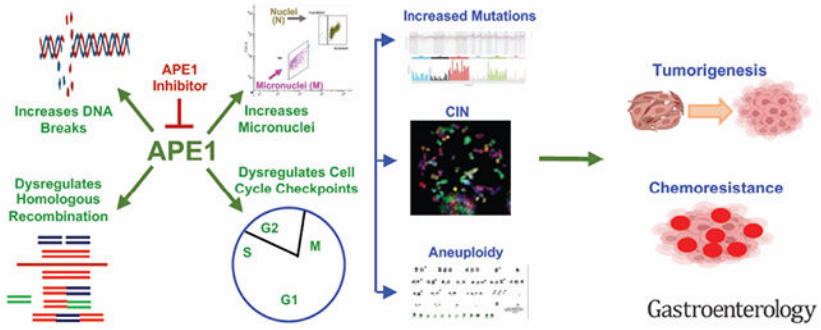
Availability of supporting data: Supplementary data file is provided with manuscript. WGS data is being deposited on NCBI Submission Portal and SNP data being deposited at publicly available website Harvard Dataverse.

Results: Expression of four deoxyribonucleases correlated with genomic instability in six human cancers. Functional screens of these genes identified APE1 as top candidate for further evaluation. APE1-suppression in EAC, breast, lung and prostate cancer cell lines caused: 1) Cell cycle arrest; 2) Impaired growth and increased cytotoxicity of cisplatin in all cell lines/types and in mouse model of EAC; 3) inhibition of homologous recombination (HR) and spontaneous and chemotherapy-induced genomic instability. APE1-overexpression in normal cells caused a massive chromosomal instability leading to their oncogenic transformation. Evaluation of these cells by WGS demonstrated the acquisition of changes throughout genome and identified HR as the top mutational process.

Conclusions: Elevated APE1 dysregulates HR and cell cycle contributing to genomic instability, tumorigenesis and chemoresistance, and its inhibitors have potential to target these processes in EAC and possibly other cancers.

Graphical Abstract



Lay Summary:

We demonstrate that inhibitors of a gene “APE1” can potentially treat cancer without harming genetic material (DNA). They can also increase efficacy of chemotherapy while reducing its toxicity to DNA.

Keywords

APE1; Homologous Recombination; Genomic Instability; Chemoresistance; Tumorigenesis

INTRODUCTION

Most cancers display diverse genomic aberrations[1–6] which evolve over time[3,7–9], indicating a marked instability at DNA and chromosomal levels[1–13]. Genomic instability is a relatively early event during oncogenesis and has been observed even in pre-neoplastic lesions[3,7,8]. Ongoing changes at DNA and chromosome levels provide new characteristics for growth and survival as well as enable affected cells to overcome immune surveillance[14] and contribute to disease progression[11,13,15]. Genomic instability underlies clonal evolution and tumor heterogeneity, and increased tumor heterogeneity can in turn lead to chemoresistance and relapse[16–17].

Esophageal adenocarcinoma (EAC), a cancer associated with gastroesophageal reflux, arises from Barrett's esophagus (BE), a precancerous condition which progresses to cancer through advancing stages of dysplasia[18]. EAC genome is strikingly aberrant[19]. Genomic instability, which exists in EAC at precancerous stage[3,8,18–24], seems to increase over time[3,23] and contribute to development of cancer and its progression. Consistently, the cancer is mostly chemoresistant[25] and associated with poor prognosis[26].

Rate of mutation in lung cancer has been estimated to be the second highest among cancers, indicating a striking genomic instability which gives rise to a heterogenous genetic landscape[27]. A signature, comprised of seven long non-coding RNAs, which correlated with genomic instability, was able to prognosticate overall survival of lung adenocarcinoma patients[28]. A striking genomic instability, as evident from complex genomic aberrations including chromothripsis, detected in prostate cancer patients has been linked to cancer progression[29]. Genomic instability has also been implicated in etiology and progression of breast cancer[30]. Defects in the pathway intermediates ensuring DNA repair and proper segregation of chromosomes seem to be among prominent mechanisms contributing to genomic instability in breast cancer[31]. Luminal B tumors of breast, that are positive for estrogen receptor but negative for progesterone receptor, have increased genomic instability and demonstrate increased growth rate and tendency to develop resistance to tamoxifen[32]. Similarly, it has been demonstrated that a genomic instability-related score calculated based on copy number alterations can predict prognosis in luminal A breast cancer[33]. It is now becoming quite evident that genomic instability is a promising target in cancer[34,35].

Chromosomal instability has been implicated in cancer progression and development of resistance to treatment and seems to associate with poor clinical outcome[13]. Identification of genes and pathways which drive genomic instability and evolution will not only improve our understanding of the oncogenic process but will help develop better strategies to treat and/or prevent cancer.

Cells in our body are constantly exposed to DNA-damaging agents (of exogenous and endogenous origin) which cause a variety of DNA lesions on daily basis[36,37]. In normal cellular condition, multiple DNA repair and related pathways coordinate to accurately repair these lesions in a timely fashion. Homologous recombination (HR), the most precise DNA repair system[38,39], contributes to repair of several types of DNA lesions and thus ensures the maintenance of genomic integrity and stability[40]. Accurate repair of DNA lesions is dependent on intact cell cycle checkpoints, especially G2, because in this phase of cell cycle the HR can utilize the sister chromatid as template to ensure error-free repair of DNA lesions[41–43]. However, error-free nature of HR also depends on its strict regulation. Dysregulation of HR, whether its reduced or increased activity, poses risk to genome stability[42]. Our data in EAC and multiple myeloma demonstrate that spontaneously elevated HR activity contributes to genetic instability[11–12], drug resistance[11] and tumor growth[44].

Purpose of this study was to identify genes driving genomic evolution in EAC and possibly other solid tumors. Since DNA breaks are required for rearrangements to take place, we hypothesized that elevated deoxyribonuclease activity drives genomic instability in cancer. Used an integrated genomics approach, we identified four deoxyribonucleases

whose expression correlated with genomic instability in six human cancers including EAC. Functional validation of these nucleases identified APE1 to have the strongest overall impact on genome stability and growth of cancer cells. APE1 (apurinic/apyrimidinic endonuclease 1) contributes to base excision repair by cleaving the DNA at 5' of abasic site[45]. Loss of a base occurs frequently in a cell[46–48]. However, thousands of such lesions generated on daily basis are fixed because of an efficient repair system[49–50]. Our data in multiple myeloma has demonstrated that APE1 also regulates HR through regulation of RAD51[51]. In this study, using cancer and normal cell types as well as in vitro and in vivo model systems, we demonstrate that elevated APE1 dysregulates HR and G2/M checkpoint, driving genomic instability, tumorigenesis and chemoresistance in cancer. Inhibitors of *APE1* have potential to inhibit growth and increase cytotoxicity of chemotherapeutic agents while minimizing spontaneous and chemotherapy-induced genomic instability in EAC and other solid tumors.

RESULTS:

Identification and functional validation of deoxyribonucleases correlating with genomic instability:

Genomic instability is a prominent feature of cancer cells. Since DNA must be cut and/or processed for genomic rearrangements to take place, we hypothesized that dysregulated nuclease activity mediates genomic instability in cancer. Consistent with this hypothesis, the evaluation of γH2AX expression (a marker of DNA breaks) in nine cancer cell lines representing five human cancers indicated that spontaneous DNA breaks are increased in cancer relative to three normal cell types (Supplementary Figure 1). To identify the nucleases contributing to DNA breaks and instability, we integrated gene expression and copy number data of six human cancers from TCGA (Figure 1a). **Identification:** First, genomic instability in each patient sample in each cancer was assessed by counting total copy number events per patient. These data from triple negative breast cancer patients are shown as example in Figure 1a whereas data from other cancer types shown in Supplementary Figure 2. Integration of genomic instability data with expression data identified deoxyribonuclease genomic instability signature (D-GIS) correlating with genomic instability in six human cancers (Figure 1a, panels II-III). D-GIS genes (APE1, EXO1, FEN1 and EME1) which seem to be functionally linked (Supplementary Figure 3A) are discussed in supplementary section. **Validation:** Elevated expression of this gene signature correlated with poor overall survival in pancreatic, lung and an esophageal adenocarcinoma (EAC) dataset (Supplementary Figure 3B). The suppression of these genes (using esiRNAs; Table 1) inhibited whereas overexpression (using overexpression plasmids; Table 2) increased genomic instability (as assessed by micronucleus assay) as well as growth rate of EAC cells (Figure 1b and Supplementary Figure 4; **details in** Supplementary Results). Overall, these data functionally validated D-GIS genes and identified APE1 as the top gene whose suppression or overexpression had the strongest impact on genomic instability and growth in EAC cells (Supplementary Table 3). Therefore, APE1 and its inhibitor were investigated further.

APE1 is overexpressed in several solid tumors and contributes to genomic instability.

Evaluation in TCGA datasets of six human cancers (EAC, esophageal adenocarcinoma; HPBC, hormone positive breast cancer; TNBC, triple negative breast cancer; LUAD; lung adenocarcinoma; PRAD, prostate adenocarcinoma; COAD, colon adenocarcinoma) demonstrated that relative to corresponding normal samples, APE1 was significantly overexpressed in tumor samples ($P < 0.04$; Figure 2a). Evaluation in frozen tissue specimens of normal squamous epithelium (NSE), precancerous Barrett's esophagus (BE), dysplasia and EAC by immunohistochemistry also indicated that APE1 expression is low in normal and precancerous (BE) cells whereas elevated in dysplasia and EAC (Figure 2b, panel I). Significantly elevated APE1 expression in EAC vs. normal esophageal tissue specimens was also observed in a tissue array ($P < 0.05$; Figure 2b, panel II). Consistently, APE1 activity (as assessed by a fluorescence-based assay) was also elevated in EAC and colon cancer cell lines tested (Supplementary Figure 5). **Impact on spontaneous genomic instability:** APE1 was suppressed in cancer cell lines (**FLO-1 and OE19**, esophageal adenocarcinoma; **MCF7**, breast cancer; **A549**, epithelial lung carcinoma; **PC3**, prostate adenocarcinoma) either using lentiviral shRNAs (Figure 2c) or by treatment with inhibitor (API3) (Figure 2d) and live cell fractions evaluated for micronuclei (a marker of genomic instability). APE1-knockdow caused significant reduction (ranging from 45% to 77%; $p < 0.05$) in genomic instability in all five cell lines tested (Figure 2c). Consistently, the treatment with API3 in FLO-1, OE19, MCF7, A549 and PC3 cells significantly reduced genomic instability by 68%, 39%, 72%, 44% and 59% respectively ($p < 0.05$; Figure 2d). Expression of APE1 also significantly correlated with a chromosomal instability signature in EAC and colon cancer patient datasets (Supplementary Figure 6) **Impact on chemotherapy-induced genomic instability:** Cancer cells were treated with API3, cisplatin (CIS) or combination and live cell fractions evaluated for micronuclei. Treatment with cisplatin caused increase in genomic instability in OE19 and A549 cells by 5.6-fold and 1.6-fold, respectively, whereas addition of API3 inhibited/prevented this increase (Figure 2e), indicating that suppression of APE1 inhibits spontaneous and chemotherapy-induced genomic instability in cancer cells.

APE1 contributes to increased DNA breaks, RAD51 expression and homologous recombination (HR). Impact on DNA breaks:

Cancer cell lines (FLO-1 and OE19, esophageal adenocarcinoma; A549, epithelial lung carcinoma; MCF7, breast cancer) in which APE1 was suppressed by inhibitor (API3) or knockdown were treated with cisplatin and live cell fractions evaluated for γ-H2AX (marker of DNA breaks) by Western blotting; GAPDH or both GAPDH and Histon H3 (a nuclear protein) were used as loading controls. Treatment with cisplatin caused substantial increase in DNA breaks whereas API3 inhibited spontaneous as well as cisplatin-induced DNA breaks in all cancer cell lines tested (Figure 3a I). Transgenic suppression of APE1 also inhibited cisplatin-induced DNA breaks in both cell lines (OE19 and A549) tested (Figure 3a II). **Impact on HR:** Based on our data in multiple myeloma demonstrating that APE1 regulates HR[51], we investigated the impact of APE1 inhibition on HR in solid tumor cells. Treatment with API3 inhibited RAD51 promoter activity (Figure 3b - I), RAD51 expression (Figure 3b - II) and HR activity (Figure 3b - III) in EAC (FLO-1) cells. Consistently, APE1 knockdown (shown in Figure 3c - I) reduced RAD51 expression (Figure 3c - II), its

phosphorylation (Figure 3c - III) and HR activity (Figure 3c - IV) in EAC cells. Inhibition of HR activity was also observed in other cell lines (representing four solid tumors) following APE1-suppression by inhibitor (API3) (Figure 3d; $p<0.05$) or knockdown (Supplementary Figure 7; *$p<0.05$; **$p<0.01$; ***$p<0.005$). Functional link between APE1 and HR pathway was further supported by mass spectrometry data demonstrating that several HR proteins (including RPA1[52], WRN[53], DHX9[54], ILF2[55] and YBX1[55]) were among top interactors of APE1 in EAC cells (Supplementary Figure 8). APE1 expression also significantly correlated with HR gene signature in EAC and colon cancer patient datasets (Supplementary Figure 9). Overall, these data demonstrate that elevated APE1 contributes to increased DNA breaks and HR in EAC and other solid tumors.

APE1 inhibition impairs growth and increases cytotoxicity of chemotherapeutic agent *in vitro* and *in vivo*. Impact on growth and colony formation.

APE1 was suppressed in cancer cell lines-EAC (FLO-1, OE19), breast cancer (MCF7), epithelial lung carcinoma (A549) and prostate adenocarcinoma (PC3) using shRNAs. Evaluation at day 7 after selection revealed that relative to control cells, the knockdown of APE1 in FLO1, OE19, MCF7, A549 and PC3 cells was associated with reduction in cell viability by 47%, 70%, 56%, 43% and 76%, respectively ($P < 0.02$; Figure 4a). APE1 inhibition, by inhibitor or knockdown, strongly impaired colony formation in all five cell lines (Supplementary Figure 10). **Impact on cytotoxicity of chemotherapeutic agents.** Cancer cell lines (MCF7, A549, FLO-1, OE19 and PC3) were cultured in the presence of APE1 inhibitor (API3), alone or in combination with cisplatin for 48 h, and cell viability assessed. API3 potentiated cytotoxicity of cisplatin (Figure 4b). Combination index plots show that increase in cytotoxicity by combination treatment was mostly synergistic or additive in all cancer cell lines tested (Supplementary Figure 11A). Evaluation in EAC (FLO-1, OE19) and breast cancer (MCF7) cell lines demonstrated that API3 also synergistically increased cytotoxicity of Olaparib (PARP; poly ADP ribose polymerase inhibitor) in all three cell lines tested (Figure 4C, Supplementary Figure 11B).

Impact on tumor growth and cytotoxicity of cisplatin in vivo.

EAC (OE19) cells were injected subcutaneously in SCID mice and following the appearance of tumors, mice treated with vehicle control, API3, cisplatin or combination of both. Relative to average tumor size in control mice, the tumor size in mice treated with API3, cisplatin and combination reduced by 48.8% ($p=0.0018$), 59.3% ($p=0.00035$) and 76.5% ($p=4.49E-05$), respectively (Figure 4D). Average tumor volume in mice treated with combination of both drugs was significantly smaller than treatment with either drug alone ($p<0.001$) (Figure 4D). Overall, these data show that APE1 inhibitor impairs cancer cell growth in vitro and in vivo and synergistically increases the efficacy of chemotherapeutic agent.

APE1 overexpression in normal cells dysregulates homologous recombination (HR) and genome stability.

Normal primary human esophageal epithelial cells (HEsEpiC) were transfected with control plasmid (C) or the plasmid carrying APE1 gene under a strong promoter to overexpress

APE1 (APE1O). **DNA breaks and HR:** APE1-overexpression (shown in Figure 5a I) was associated with increased DNA breaks as assessed by Comet assay (Figure 5A II). APE1-overexpression also led to increased RAD51 expression (Figure 5A I) and HR activity (Figure 5A III). **Karyotypic instability:** APE1-overexpressing (APE1O) cells were cultured for 60 days, and chromosomes visualized by spectral karyotyping. Control cells had near diploid karyotype with mitotic index (MI) of 0.16% and 0.2 aberrations/cell (Figure 5B I), whereas APE1O cells were near tetraploid with MI of 3.9%, 16 aberrations/cell, 4 copies of C-myc/cell and several chromosomal abnormalities, indicating a striking karyotypic instability (Figure 5B II–III). Consistently, APE1-overexpression in these HEsEpiC as well as normal fibroblasts was also associated with centrosome amplification (Supplementary Figure 12). **Whole genome sequencing demonstrates mutational instability:** Genomic impact of APE1-overexpression in normal cells was evaluated by whole genome sequencing. Genome of day 0 cells was used as baseline to identify the mutations acquired by control and APE1-overexpressing cells over a period of 60 days in culture. Although, control cells had only 83 new mutations, those acquired by APE1-overexpressing cells were > 25,000 (Figure 5C I). Removal of known SNPs from these data identified 3500 mutations unique to APE1-overexpression. These mutations were further investigated for subtypes and signatures of underlying mutational processes as described by us previously[10,56]. Most of the mutations caused by APEI-overexpression were C>T substitutions which were followed by T>C and then C>A (Figure 5C II). Investigation of mutational signatures identified Signature 3, indicative of HR dysfunction, as the top mutational process activated by APE1 (Figure 5C III). **Copy number changes:** DNA samples from control and APE1-overexpressing cells cultured for a period of 60 days were also investigated for copy number alterations using single nucleotide polymorphism arrays. Relative to day 0 cells, the copy number events acquired in control and APE1-overexpressing cells were 66 and 5734, respectively (Supplementary Figure 13). These data are commensurate with that observed by whole genome sequencing.

APE1-induced genomic instability can predispose normal cells to oncogenic transformation:

Consistent with increased DNA and karyotypic instability, the APE1-overexpressing normal cells (HEsEpiC) changed in morphology (Figure 6A) and had significantly increased growth rate relative to control plasmid-transfected cells (Figure 6B). **Tumors in xenograft model:** When injected subcutaneously in SCID mice, control cells (injected on left side of each mouse) did not form tumors, but APE1-overexpressing cells (injected right side of each mouse) formed tumors (Figure 6C). Investigation of tumors from mice demonstrated further chromosomal rearrangement in vivo (Supplementary Figure 14; details in Supplementary Results). These data demonstrate that elevated APE1 activity drives genomic instability, predisposing normal cells to tumorigenesis.

APE1 contributes to cell cycle progression:

APE1 was either suppressed in EAC (FLO-1) cells (using APE1 inhibitor; API3) or overexpressed in normal esophageal epithelial cells and impact on gene expression evaluated. Investigation of the pathways that were downregulated in API3-treated EAC cells whereas upregulated in APE1-overexpressing normal cells, identified G2/M checkpoint as

the most significant common pathway impacted (Figure 7a I). APE1 inhibition in seven cancer cell lines (representing four cancers) affected cell cycle progression at multiple levels with some of the changes as cell type specific. A relatively consistent observation was increase in fraction of cells in G2 phase of cell cycle. Relative to control cells, the treatment of MDA453, FLO-1, OE33, PC3, OE19, MCF7 and A549 cells led to 3.2, 2.6, 1.7, 1.6, 1.4, 1.4 and 1.2-fold increase in fraction of cells in G2, respectively; increase in MDA453, FLO-1, PC3 and A549 was significant ($P \leq 0.05$; Figure 7b). In addition, S phase fraction decreased in API3-treated MCF7, PC3 and MDA453 cell lines whereas slightly increased in OE33 cells. In PC3 cells sub G1 fraction also increased substantially whereas hyperploid population peak increased slightly (Figure 7b). These data suggest role of APE1 in cell cycle progression at multiple levels with a more prominent impact on G2 checkpoint in several cell types.In summary, we show that elevated APE1 dysregulates of HR and cell cycle contributing to genomic instability, tumorigenesis and oncogenesis (model in Supplementary Figure 15).

DISCUSSION:

Genomic instability, a common feature of precancerous and cancer cells[1–13], leads to ongoing acquisition of changes at both the DNA and chromosome level. These changes not only provide new characteristics for growth and survival of these cells but also contribute to escape of aberrant cells from immune surveillance[14], development of resistance to cancer treatment[11,13,15], progression to advanced stages of disease[56] and ultimately impact the clinical outcome of disease[13]. A striking genomic instability observed in EAC and its premalignant states[3,8,18–24] could be attributed to its chemoresistant[25] nature. A marked genomic instability has also been observed in breast[30], lung[27] and prostate[29] cancers and is attributed to disease progression[29–30] and poor clinical outcome[28]. Consistently, it has been proposed that genomic instability is a promising target in cancer[34,35]. The purpose of this study was to identify genes driving genomic instability in EAC as well as other solid tumors. Since DNA must be cut or broken for genomic rearrangements to take place, we hypothesized that dysregulated deoxyribonuclease activity mediates genomic instability in cancer. Consistent with this hypothesis, the evaluation of γH2AX expression in nine cancer cell lines representing five solid tumors demonstrated that spontaneous DNA breaks are increased in cancer relative to normal cell types (Supplementary Figure 1). To find deoxyribonucleases which contribute to spontaneous DNA damage and instability, we used an integrated genomics strategy similar to that described by us previously[57]. Although ribonucleases also contribute to genomic instability, in this study, we focused on deoxyribonucleases because of their ability to directly impact DNA. Since topoisomersases can have additional (helicase and ligase) activities, they were also not included in this analysis. We thus identified a four gene deoxyribonuclease signature correlating with genomic instability in patient (TCGA) datasets representing six solid tumors (adenocarcinomas of esophagus, lung, prostate, stomach, pancreas and triple negative breast cancer). Elevated expression of this signature also correlated with poor overall survival in pancreatic, lung and one of the esophageal adenocarcinoma datasets, establishing the functional relevance of these nucleases. Moreover, functional studies in EAC cells confirmed that inhibition of these nucleases reduced whereas their overexpression increased genomic

instability as well as growth rate of these cancer cells. Overall, APE1 was identified as the top gene whose suppression as well as overexpression had the strongest impact on genomic instability and growth of EAC cells. Therefore, APE1 and its inhibitor were investigated in detail for impact on various parameters of genome stability and growth in cancer cell lines representing four different solid cancers including EAC.

APE1 has important roles in the maintenance of genome stability and growth of cells (see discussion section of Supplementary Material for detailed information). Investigation of patient samples in TCGA dataset showed that relative to normal tissues the expression of APE1 is significantly elevated in human cancer specimens of hormone positive and triple negative breast cancers and adenocarcinomas of esophagus (EAC), lung, prostate and colon. Immunohistochemical evaluation of patient specimens also demonstrated that expression of APE1 is elevated in dysplasia and EAC relative to that in normal and precancerous cells. Consistent with our data, elevated APE1 expression has also been reported by other investigators in cervical[62], ovarian[63], rhabdomyosarcomas[64], prostate[65], and germ cell tumors[66] thus supporting its potential to be used as a therapeutic target.

We hypothesized that dysregulated deoxyribonuclease activity increases DNA breaks leading to genomic instability. Consistent with this hypothesis, using multiple cell and tumor types and evaluation by different platforms (micronucleus assay, SNP profiling, whole genome sequencing and spectral karyotyping), we demonstrate that suppression of APE1 in cancer cells reduces spontaneous and chemotherapeutic agent-induced genomic instability, whereas its overexpression in normal cells causes a striking genomic instability at both DNA and chromosome level. To our knowledge, this is first report demonstrating acquisition of copy number, mutational and karyotypic changes over time by APE1.

Our previous investigation in EAC and multiple myeloma (MM) model systems have demonstrated that homologous recombination (HR) is elevated/dysregulated and attributed to acquisition of genomic rearrangements over time[11–12], development of drug resistance[11] and growth of cancer cells in vivo[44]. Our investigation in MM has also demonstrated that APE1 impacts HR through regulation of RAD51[51]. However, dysregulated APE1 activity can also impact HR through induction of DNA breaks. We, therefore, investigated the impact of APE1 modulation on DNA breaks and HR activity in cancer and normal cells. Overall, our data indicate that increased APE1 expression contributes to spontaneous and chemotherapy-induced DNA breaks in affected cells.

Inhibition of APE1 (by its inhibitor or knockdown) in cancer cell lines (representing EAC, prostate, breast and lung cancers) inhibited, whereas its overexpression in normal cells significantly increased, HR activity. These data are consistent with our data in MM[51] demonstrating that APE1 regulates HR. Moreover, identification of APE1-interacting proteins in EAC cells by mass spectrometry and evaluation of top interactors by protein-protein network analysis identified several proteins with known function in HR including RPA1[52], WRN[53], DHX9[54], ILF2[55] and YBX1[55]. More importantly, the evaluation of the impact of APE1-overexpression in normal cells by whole genome sequencing identified HR as the top mutational signature (Figure 5). Since mutational signatures point to the

underlying mutational processes[68–69], these data provide further evidence for the role of HR in APE1-mediated genomic instability.

APE1-knockdown reduced growth rate and impaired colony formation in all five cell lines representing four solid tumors (breast cancer, epithelial lung carcinoma and adenocarcinomas of esophagus and prostate). Treatment with APE1 inhibitor also inhibited growth of cancer cells and potentiated cytotoxicity of cisplatin in all five cell lines and in a subcutaneous model of EAC. Consistent with our findings, it has been shown that APE1 contributes to growth of pancreatic cancer cells[70] and its expression in cervical cancers is commensurate with radiosensitivity[71]. Moreover, a role of APE1 in the regulation of genes involved in chemoresistance has also been proposed[72].

Consistently, the overexpression of APE1 in normal esophageal epithelial cells (HEsEpiC) dysregulated HR and cell cycle leading to genomic instability and oncogenic transformation. Here, we would like to clarify that as per technical service team of supplier (ScienCell) and our own evaluation for relevant markers (Supplementary Figure 16), HEsEpiCs are a mixture of squamous and columnar cells and may not be considered as exact control for EAC. In fact, to our knowledge the exact normal cell of origin of EAC is not available. Our purpose to use HEsEpiCs was to demonstrate the consequence of APE1-overexpression in a normal esophageal epithelial cell model system. Consistent with our results in HEsEpiCs, we now also have preliminary evidence of APE1-induced genomic instability and tumorigenesis in transgenic animal models (not shown).

Investigation of common pathways impacted by APE1 suppression in EAC and its overexpression in normal esophageal cells by RNASeq, identified G2/M checkpoint as the most significant common pathway impacted (Figure 7a). Consistently, the treatment with APE1 inhibitor, induced G2/M arrest in all five human cancer cell lines representing four solid tumors. Functional role of APE1 in G2/M progression could be attributed to its ability to impact growth and genomic instability in cancer cells. Cell cycle checkpoints ensure repair of DNA damage prior to DNA replication (in G1) and segregation (in G2), thus ensuring maintenance of genomic integrity. Moreover, G2 is the phase where HR can utilize sister chromatid as template to ensure precise repair of the damage. Thus, defective G2/M checkpoint[73] and dysregulated HR[11–12,42] plays significant role in genomic rearrangements and evolution in cancer. An important question here could be that how APE1 could inhibit genomic instability while increasing cytotoxicity of cisplatin. Please see Discussion Part of Supplementary Material for clarification.

Conclusion

We conclude that APE1, identified as part of four gene deoxyribonuclease signature, dysregulates HR and G2/M checkpoint driving genomic instability, tumorigenesis and chemoresistance in EAC and other solid tumors **(summarized in** Supplementary Figure 15). Therefore, inhibitors of APE1, alone and/or in combination with other agents, have potential to make EAC and possibly other cancers static by targeting HR, cell cycle progression and genomic instability.

MATERIALS AND METHODS:

Patient datasets and specimens:

The Cancer Genome Atlas (TCGA) data were used to investigate gene expression and copy number events in six human cancers including esophageal adenocarcinoma (EAC; details in supplemental section). De-identified specimens of normal esophageal epithelial squamous (NES), Barrett's esophagus (BE, a precancerous lesion for EAC), dysplasia and EAC were provided by our collaborator Dr. Hiroshi Mashimo who has an active protocol (R&D #2490, ID #1578027) at Boston VA Healthcare Center, MA. Esophageal cancer progression tissue arrays were purchased from US Biomax, Inc. (Rockville, MD).

Antibodies:

(Described in Methods section of Supplementary Material).

Overexpression and knockdown plasmids and siRNAs:

(see Methods section of Supplementary Material).

Identification of a deoxyribonuclease signature correlating with genomic instability.

We hypothesized that if elevated expression of a gene correlates with increased genomic instability in multiple human cancers, it could be a potential driver of genomic evolution. Since dysregulated nuclease activity can directly impact genome stability, we focused on deoxyribonucleases (excluding topoisomerases) for this study. To identify the nucleases whose expression correlated with genomic instability, we used following stepwise process: **1)** Investigated gene expression in normal and tumor samples for six human cancers in TCGA dataset and identified deoxyribonucleases (excluding topoisomerases); **2)** Assessed genomic instability in patient samples by counting total number of copy events in each patient; **3)** Integrated genomic instability data with expression data to identify deoxyribonucleases whose expression correlated with genomic instability; 4) Four gene nuclease signature was validated in functional screens. Apurinic/apyrimidinic nuclease 1 (APE1), demonstrating the strongest overall impact on genomic instability and growth, was evaluated in detail for its role in genomic instability, cell cycle and oncogenic process.

Cell types:

(See Supplementary Material)

Cell Viability:

Cell Titer-Glo Luminescent Viability Assay kit (Promega Corporation, Madison, WI) was used to assess cell viability.

Modulation of gene expression/function.

(See Supplementary Material)

Gene Expression Analysis and Biostatistics.

Total RNA was isolated utilizing an “RNeasy” kit (Qiagen Inc., USA) and gene expression profile was evaluated using Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) as described by us previously[11,12,75].

Homologous Recombination and Related Assays:

Homologous recombination (HR) activity was assessed using the plasmid-based functional assay described previously[12,44]. Homologous strand exchange (SE) activity was measured using a fluorescence-based assay as reported by Huang et al[77].

Detection of DNA breaks and end resection.

DNA breaks were assessed from γ-H2AX expression (marker of DNA breaks) using Western blotting or by Comet assay, a gel-based method to visualize DNA breaks. Comet assay was done using OxiSelect™ Comet Assay Kit (Cell Biolabs Inc., San Diego, CA) as described by us previously[75]. DNA end resection was assessed by investigating the phosphorylation of RPA32 on ser4 and ser8[78–79].

Investigation of protein-protein interactions, expression and phosphorylation levels.

(See Methods section of Supplementary Material).

Cell cycle analysis:

Cells were washed twice with PBS and fixed in 95% ethanol. Cells were pelleted, washed and resuspended in propidium iodide/RNAse solution (Life Technologies) and investigated using flow cytometry. Data was analyzed using FLOJO software.

Investigating DNA/chromosomal instability and evolution.

Genomic instability and evolution were investigated using multiple different methods as described below:

- ***Micronucleus assay.*** Micronuclei (marker of genomic instability[67]) were investigated by a flow cytometry-based assay using a kit. For this evaluation, live cell fractions were used as previously reported by us[51,57].
- ***Single nucleotide polymorphism (SNP) arrays and Whole genome sequencing (WGS).*** Cells were cultured for different durations, and genomic DNA from live cells purified and analyzed using either SNP6.0 arrays (Affymetrix) or WGS. Genome of “day 0” cells (harvested and saved in the beginning of each experiment) was used as baseline to identify changes in control and transgenically-modified/treated cells during their growth in culture. WGS and SNP data were analyzed as reported by us previously[11–12,57].
- ***Chromosomal instability*****:** Karyotypic changes were evaluated using multicolor fluorescence in situ hybridization (details in Methods section of Supplementary Material).

In vivo evaluations:

Impact of APE1 inhibitor and/or cisplatin in EAC cell line and the impact of APE1 overexpression in normal esophageal epithelial cells on tumorigenesis were monitored using SCID mice (details in Methods section of Supplementary Material).

Statistics and reproducibility (details in Methods section of Supplementary Material).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What You Need to Know:

BACKGROUND AND CONTEXT

Genomic instability or the ability to obtain ongoing genomic changes, provides new characteristics for growth and survival as well as enables affected cells to overcome immune surveillance and contribute to development and progression of cancer. Esophageal adenocarcinoma (EAC), a cancer associated with strikingly genomic instability, is mostly chemoresistant and associated with poor prognosis. Purpose of this study was to identify drivers of genomic instability and evaluate their translational significance in EAC and other solid tumors.

NEW FINDINGS

Using an integrated genomics protocol, we identified a deoxyribonuclease signature correlating with genomic instability in six human cancers. Functional validation of this signature identified apurinic/apyrimidinic nuclease 1 (APE1) as a top gene impacting genome stability and growth in esophageal adenocarcinoma (EAC) cells. APE1 was further investigated in five cell lines representing four solid tumors (EAC, breast cancer, epithelial lung carcinoma and prostate adenocarcinoma). Inhibition of APE1 in cancer cells induced cell cycle arrest, impaired their growth and increased cytotoxicity of chemotherapeutic agent in vitro and in vivo. The inhibition of APE1 also inhibited homologous recombination (HR) activity and reduced spontaneous and chemotherapy-induced genomic instability in all cancer cell types. Consistently, the overexpression of APE1 in normal human cells caused a massive chromosomal instability leading to their oncogenic transformation, and evaluation of by whole genome sequencing identified HR as the top mutational process activated in these cells. Overall, we demonstrate that elevated APE1 dysregulates HR and cell cycle thus contributing to genomic instability, tumorigenesis and chemoresistance, and its inhibitors have potential to target these processes in EAC and multiple solid tumors.

LIMITATIONS

More efficacious inhibitors of APE1 suited for in vivo utilization are required for further clinical application of these findings.

CLINICAL RESEARCH RELEVANCE and BASIC RESEARCH RELEVANCE

Inhibitors of APE1 and other nucleases identified in this study have potential to target growth as well as spontaneous and chemotherapy-induced genomic instability in EAC and possibly other cancers. Such inhibitors, alone or in combination with existing drugs, may also inhibit/reduce or delay progression of cancer, which is associated with genomic instability. APE1 and other targets identified in this study also provide model systems to study genomic instability and its mechanisms and consequences in vitro and in transgenic models.

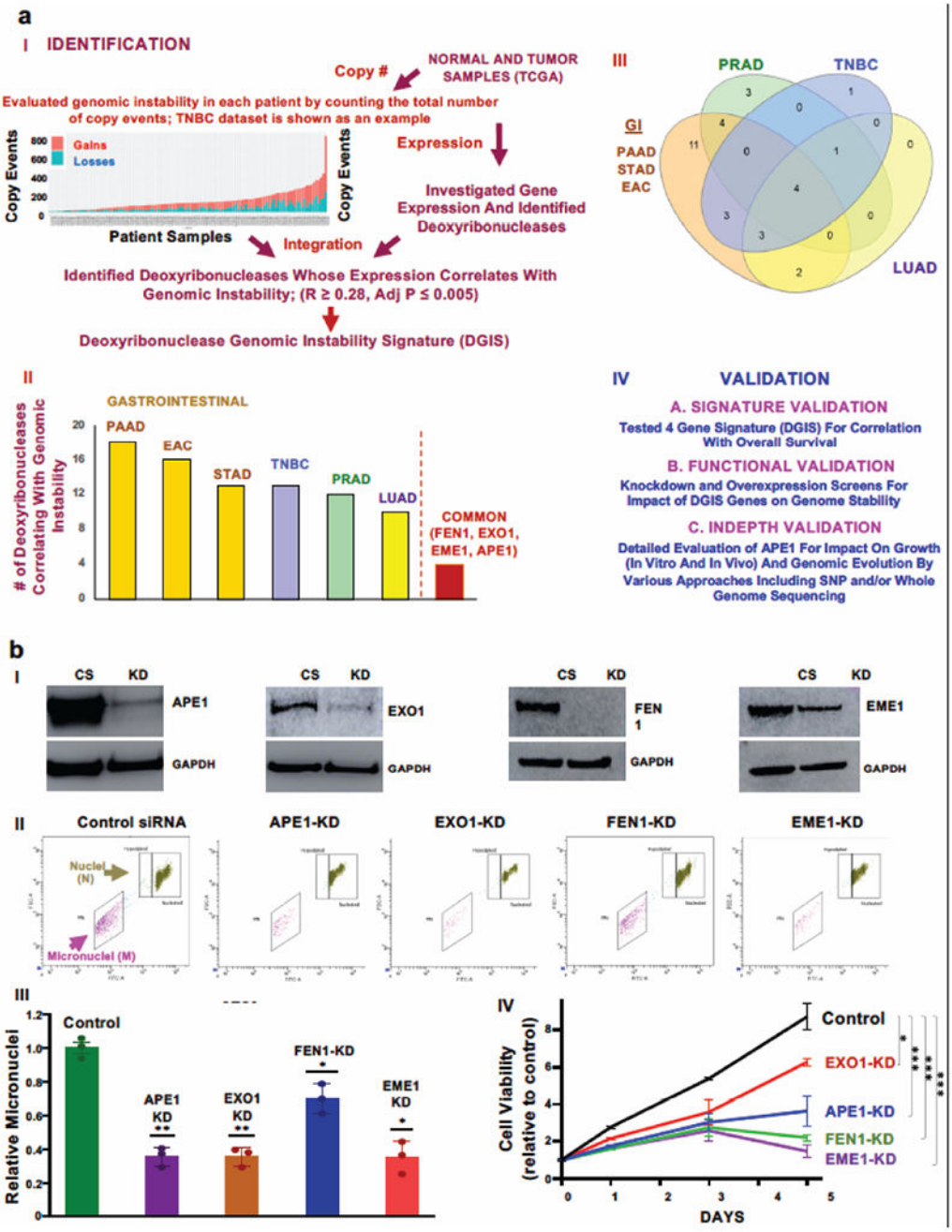

Figure 1. Identification and validation of deoxyribonucleases impacting genome stability in solid tumors.

(A) Identification: (I) Flow chart describing the stepwise process; **(II)** Bar graph showing number of deoxyribonucleases whose expression correlated with genomic instability in each cancer; **(III)** Venn diagram showing genomic instability-associated deoxyribonucleases in six human cancers. LUAD, lung adenocarcinoma; PRAD, prostate adenocarcinoma; TNBC, triple negative breast cancer; GI (gastrointestinal cancers – EAC, esophageal adenocarcinoma; STAD, stomach adenocarcinoma; PAAD, pancreatic adenocarcinoma).

(IV) Plan for functional validation of identified genes. **(B) Validation:** Genomic instability-associated deoxyribonucleases were suppressed in EAC (FLO1) cells using esiRNAs (described in Supplementary Table 1); **(I)** Western blot showing knockdown of each gene; **(II-IV)** Impact on micronuclei (marker of genomic instability). Images showing nuclei (N) and micronuclei (MN) (II) and bar graph showing relative micronuclei (III) are shown; **(IV)** Cell viability assessed using Cell Titer-Glo; CS, control esiRNA; KD, esiRNA-mediated knockdown. Error bars in panels III-IV represent SDs of triplicate experiments and two-tailed *P*-values derived by Student t-test (**P*<0.05-0.01; ***P*< 0.01-0.001; ****P*<0.001).

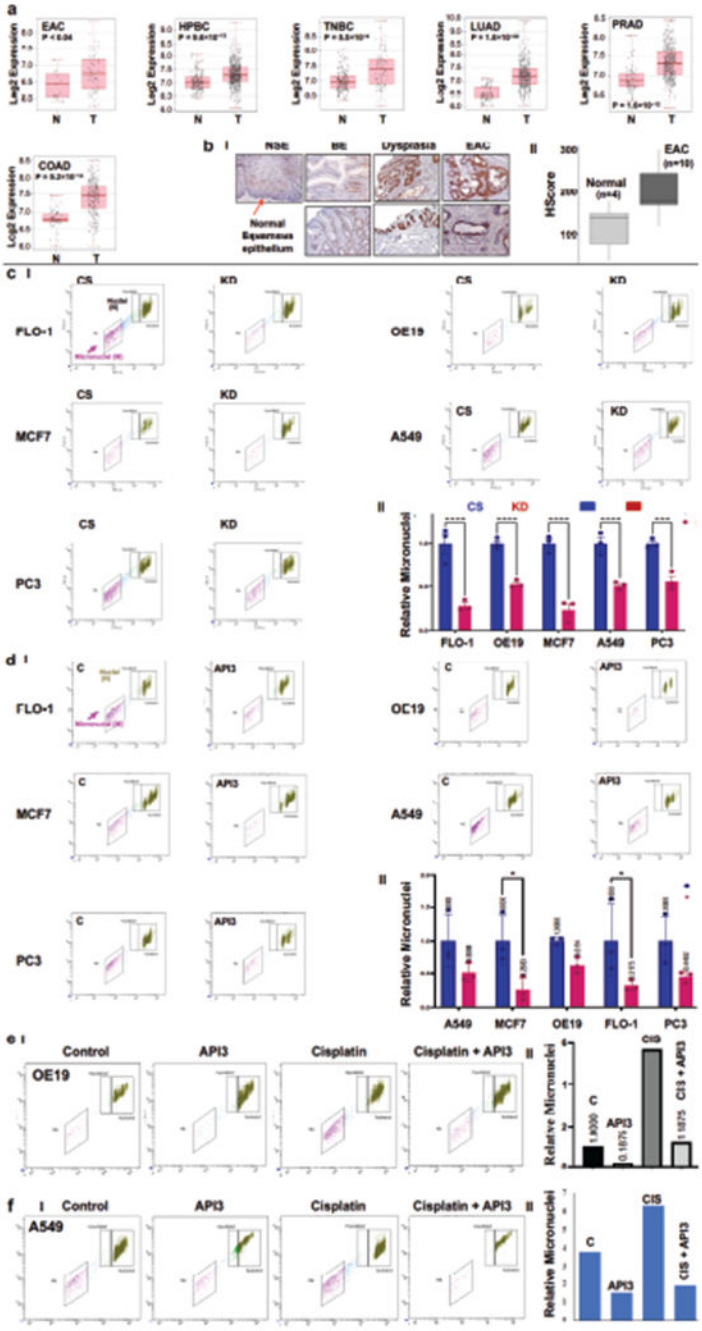

Figure 2. APE1 is overexpressed in EAC and other solid tumors and contributes to spontaneous as well as chemotherapy-induced genomic instability.

(A) Relative expression (Log2) of APE1 in TCGA patient datasets (EAC, esophageal adenocarcinoma; HPBC, hormone positive breast cancer; TNBC, triple negative breast cancer; LUAD; lung adenocarcinoma; PRAD, prostate adenocarcinoma; COAD, colon adenocarcinoma). **(B)** APE1 expression in frozen tissue specimens of normal squamous epithelium (NSE), Barrett's esophagus (BE), dysplasia and EAC detected by immunohistochemistry (panel I) and in EAC and normal esophageal specimens on a tissue

array (Biomax; panel II); **(C–D)** APE1 was suppressed in cancer cell lines (FLO-1 and OE19, esophageal adenocarcinoma; MCF7, breast cancer; A549, epithelial lung carcinoma; PC3, prostate adenocarcinoma) either using lentiviral shRNAs (described in Supplementary Table 1) (panel C) or by treatment with inhibitor (API3; 1.5 **μ**M) for 48 h (panel D) and live cell fractions evaluated for micronuclei (marker of genomic instability). Panels: Representative images showing nuclei (N) and micronuclei (MN) (I) and bar graph (II) showing relative micronuclei; error bars indicate SDs of triplicate experiment and two-tailed *P*-values derived by paired t-test (**P*<0.05-0.01; ***P*< 0.01-0.001; ****P*<0.001). CS, control shRNA; KD, APE1-knockdown; C, control (DMSO); API3, APE1 inhibitor; **(E)** Cancer cell lines, OE19 (top panel) and A549 (bottom panel) were treated with API3 (1.5 **μ**M), cisplatin (CIS; 5 **μ**M) or combination and live cell fractions evaluated for micronuclei. Representative images showing nuclei (N) and micronuclei (MN) (I) and bar graph (II) showing fold change in micronuclei, relative to control cells.

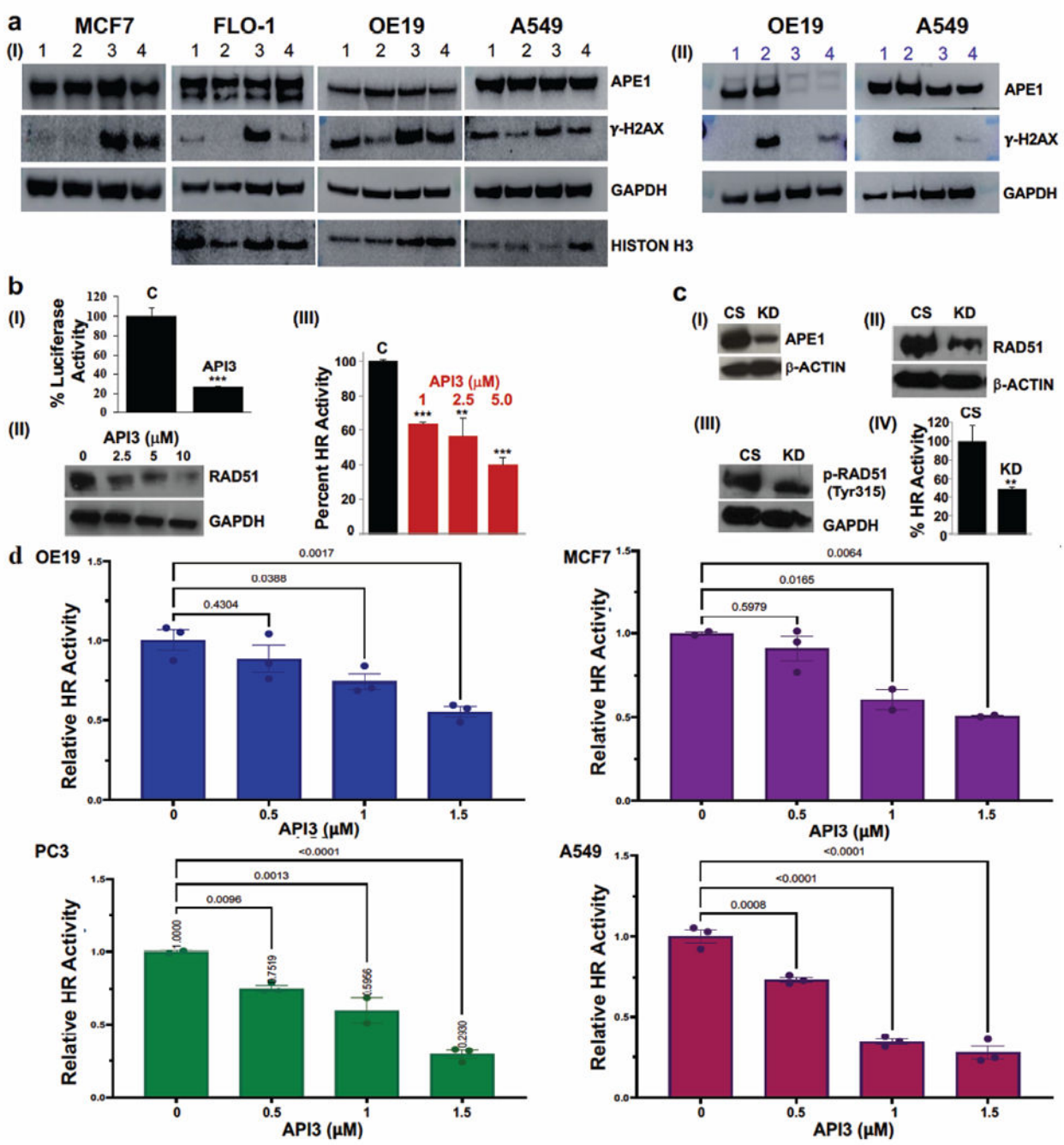

Figure 3. APE1 contributes to increased DNA breaks, RAD51 expression and homologous recombination (HR) in cancer cells.

(A) APE1 was suppressed in cancer cell lines by treatment with APE1 inhibitor (API3; 1.5 **μ**M) (I) or shRNAs (II). Cells were cultured in the presence or absence of cisplatin (5 **μ**M) and live cell fractions evaluated for **γ**-H2AX expression by Western blotting. **Lanes:** (I) 1, control; 2, API3; 3, cisplatin; 4, API3+cisplatin; (II) 1, control shRNA; 2, control shRNA+cisplatin; 3, APE1-shRNA; 4, APE1-shRNA+cisplatin; **(B)** FLO-1 cells treated with API3 were evaluated for RAD51 expression by Western blotting (I), RAD51 promoter

activity using luciferase-based assay (II) and HR activity using plasmid-based assay (III). **(C)** FLO-1 cells were treated with shRNAs (CS, control; KD, APE1-knockdown) and evaluated for expression of APE1 (I); RAD51 (II) and phosphorylated-RAD51 by Western blotting (III) and HR activity (IV); **(D)** Cancer cells were treated with API3 for 48 h and impact on HR activity determined. Error bars indicate SDs of triplicate experiments; two-tailed *P*-values for panels B-C are **P*<0.05-0.01; ***P*< 0.01-0.001; ****P*<0.001 and those for panel D shown in figure.

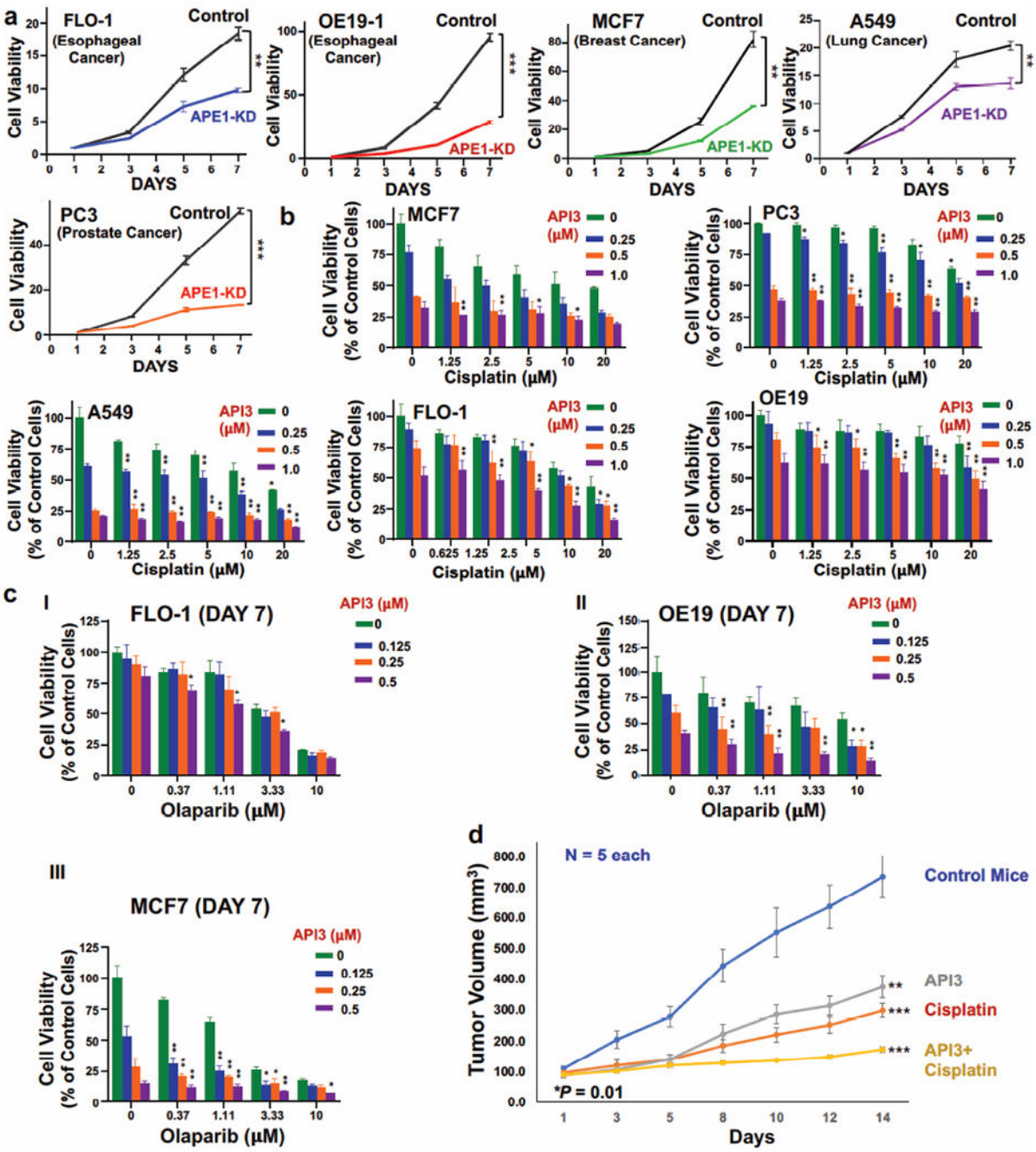

Figure 4. Suppression of APE1 inhibits growth and increases cytotoxicity of cisplatin and olaparib.

(A) APE1 was suppressed in human cancer cell lines – esophageal adenocarcinoma (FLO-1, OE19), breast cancer (MCF7), epithelial lung carcinoma (A549) and prostate adenocarcinoma (PC3) using shRNAs and impact on cell viability assessed; Control, control shRNA; APE1-KD, APE1 shRNA; error bars indicate SDs of triplicate experiment and two-tailed *P*-values (**P*<0.05-0.01; ***P*< 0.01-0.001; ****P*<0.001) show significance of difference between control and knockdown. **(B-C)** Cancer cell lines were treated with APE1

inhibitor (API3), alone and in combination with cisplatin for two days (panel B) or PARP inhibitor (Olaparib) for seven days (panel C), and cell viability assessed. Error bars represent SDs of three experiments and *P*-values (**P*<0.05-0.01; ***P*<0.01) show significance of difference between corresponding control and treated samples. **(D) *In vivo* evaluation.** EAC (OE19) cells were injected subcutaneously in SCID mice and following appearance of tumors, mice treated with either DMSO, API3 (12 mg/kg, daily for 2 weeks), cisplatin (3 mg/kg, once a week for 2 weeks) or combination of both drugs. Line plots show tumor growth (n = 5 each); error bars represent SDs. ***P* values:** **P*=0.001, combination vs. single treatments; ***P*<0.005, control vs. API3; ****P*<0.0005, control vs. cisplatin and control vs. combination.

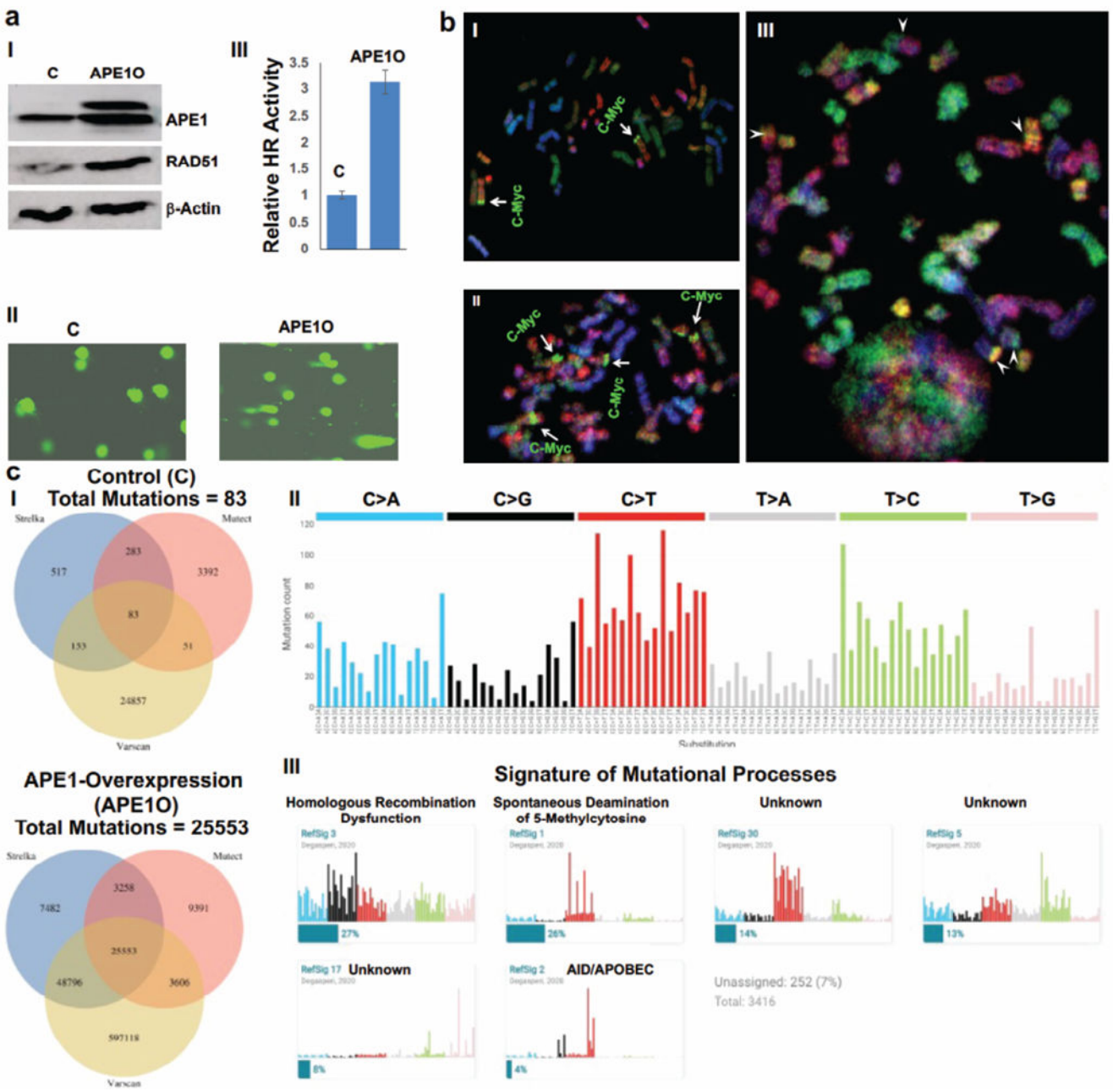

Figure 5. APE1 overexpression in normal esophageal cells induces DNA breaks, homologous recombination (HR) and chromosomal/mutational instability.

Control and APE1-overexpressing (APE1O) normal primary human esophageal epithelial cells were evaluated for various parameters of genome stability. **(A) DNA breaks and HR. (I)** Western blot showing expression of APE1, RAD51 and γ-H2AX (marker of DNA breaks) in control and APE1-overexpressing cells, evaluated right after selection; **(II)** DNA breaks assessed by alkaline Comet assay right after selection; **(III)** HR activity evaluated by plasmid-based assay; error bars represent SD of three experiments and two tailed *P* value indicates significance of difference between control and APE1-overexpression (***$P<0.001$); **(B) Impact on karyotype:** Karyotypes of control **(I)** and APE1O (**II-III**)

cells examined after 60 days in culture. Panels II and III are two different examples of karyotypes. Mitotic index (MI) of APE1O = 3.9%; 9-12 chromosome aberrations (arrowheads on representative translocations); **(C) Impact on mutational frequency evaluated by WGS:** Cells were cultured for sixty days and analyzed for new mutations, relative to baseline (day 0) cells, using WGS. **(I)** New mutations in control and APE1O cells identified by 3 different software tools; **(II)** Substitution types in APE1-overexpressing cells throughout genome are presented in context of the sequence immediately 5’ and 3’ to the mutated base; **(III)** Fraction of contribution of each mutation type at each sequence context for the mutational signatures. For investigation of mutational signatures or processes, SNPs were removed to ensure that somatic mutation callers do not confuse about the SNPs and SNVs.

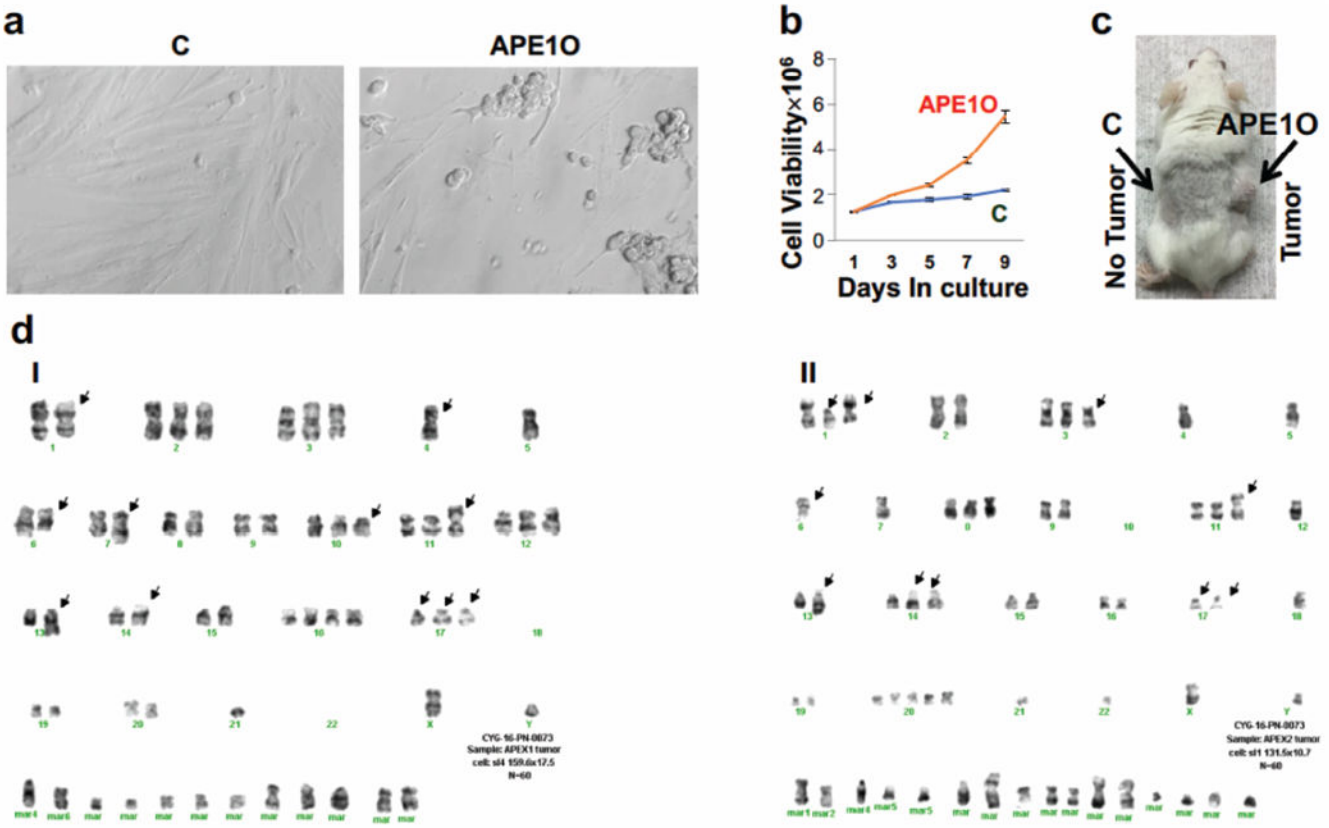

Figure 6. APE1 overexpression causes oncogenic transformation of normal human esophageal cells.

(A) Photograph of control and APE1O (APE1-overexpressing) human esophageal epithelial cells; **(B)** Cells were cultured and viable cell number assessed using Cell Titer-Glo; error bars represent SDs of triplicate experiment; Two-tailed *P* value (***$P<0.001$) indicates significance of difference between control and APE1-overexpression. **(C)** Control cells injected on left side of SCID mice did not grow as tumor whereas APE1O cells injected on right side of each mouse grew as tumor; one mouse is shown as an example; **(D)** Tumors from control and APE1O mice were evaluated for karyotype; Panels I and II are two representative karyotypes.

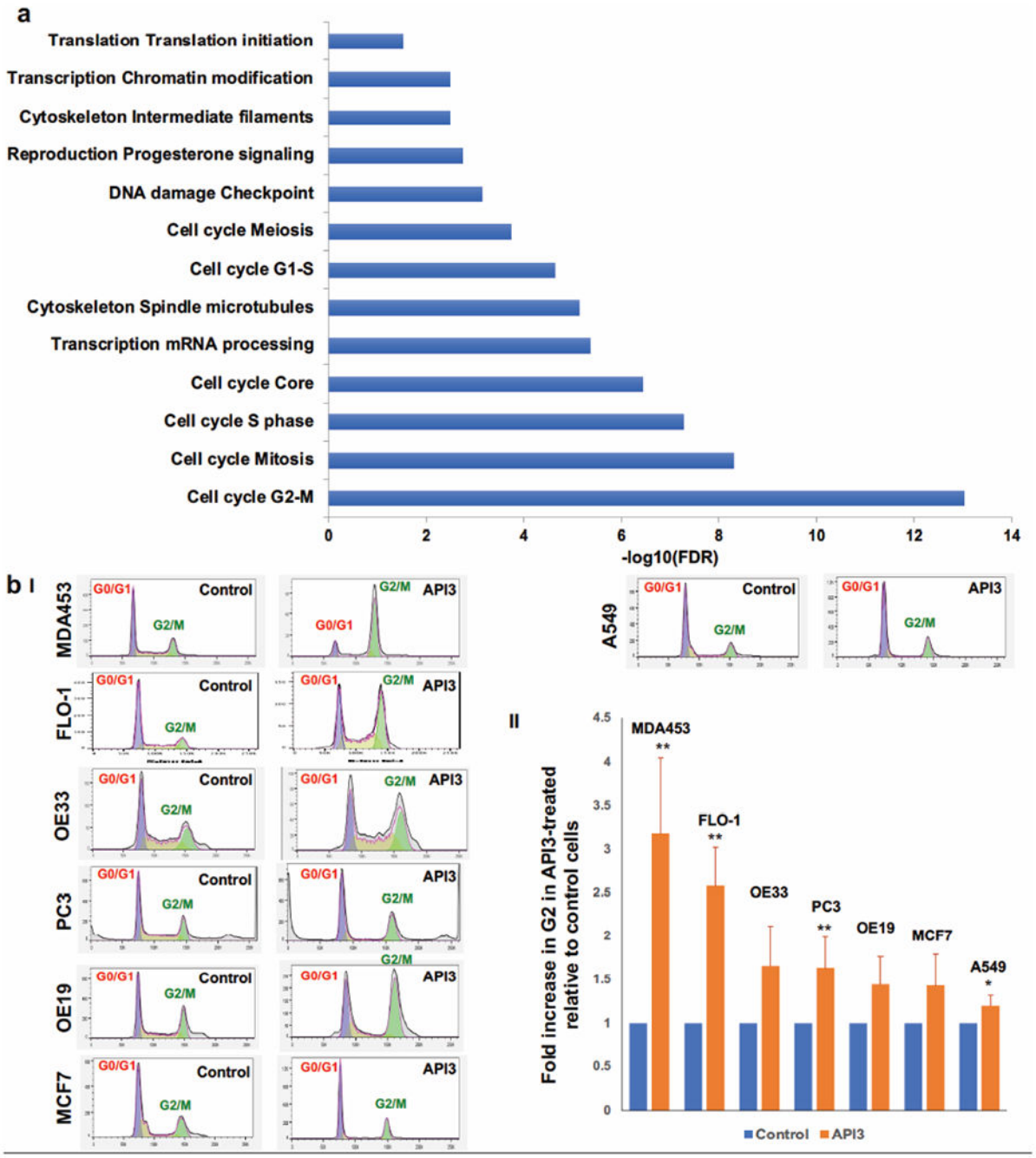

Figure 7. APE1 contributes to G2/M progression in solid cancers.

(A) Expression profile showing common pathways which are upregulated following APE1-overexpression in normal esophageal epithelial (HEsEpi) cells, whereas downregulated in EAC (FLO1) cells treated with APE1 inhibitor (API3). Human cancer cell lines - esophageal adenocarcinoma (FLO-1, OE19, OE33), breast cancer (MDA453, MCF7), epithelial lung carcinoma (A549) and prostate adenocarcinoma (PC3) were treated with API3 for 48 h and evaluated for cell cycle; **Panels: (I–IV)** Cell cycle profiles; **(V)** Bar graph showing percent increase in G2 fraction relative to control cells. Error bars represent SDs of three

experiments; one tailed *P* values (**P*=0.05; ***P*<0.05) indicate significance of increase in G2 in API3-treated relative to control cells.