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Securin induces genetic instability in colorectal cancer by inhibiting double-stranded DNA repair activity

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Genetic instability (GI) is a hallmark feature of tumor development. Securin, also known as pituitary tumor transforming gene (*PTTG*), is a mitotic checkpoint protein which is highly expressed in numerous cancers, is associated with tumor invasiveness, and induces GI in thyroid cells. We used fluorescence inter-simple sequence repeat PCR to assess GI caused primarily by DNA breakage events in 19 colorectal tumors. GI values ranged significantly, with Dukes' stage C&D colorectal tumors exhibiting greater GI and higher securin expression than Dukes' stage A&B tumors. Consistent with these findings, we observed a dose-dependent increase in GI in HCT116 cells in response to securin overexpression, as well as in non-transformed human fibroblasts. As securin has been implicated in a novel DNA repair pathway in fission yeast, we investigated its potential role in chemotoxic DNA damage response pathways in mammalian cells, using host cell reactivation assays. Securin overexpression in HCT116 cells inhibited etoposide-induced double-stranded DNA damage repair activity, and repressed Ku heterodimer function. Additionally, we observed that securin and Ku70 showed a reciprocal cytosol–nuclear translocation in response to etoposide-induced dsDNA damage. Our data suggest that, by repressing Ku70 activity and inhibiting the non-homologous end-joining dsDNA repair pathway, securin may be a critical gene in the development of GI in colorectal cancer.

Introduction

Genetic instability (GI) is implicated in both the initiation and progression of tumors (1), and may serve as a valuable molecular diagnostic and prognostic indicator (2–5). GI is apparent in colorectal adenomatous polyps as well as colorectal tumors (6,7), and has been shown to be associated with tumor stage, prognosis and response to chemo-radiotherapy in patients with colorectal cancer (8,9).

Abbreviations: DSB, double-strand break; dsDNA, double-stranded DNA; FISSR-PCR, fluorescence inter-simple sequence repeat polymerase chain reaction; GI, genetic instability; PK-DNA, protein kinase-dependent DNA repair; *PTTG*, pituitary tumor transforming gene; RLU, relative light units; VO, vector-only.

GI can be broadly classified into (i) chromosomal instability or aneuploidy, in which entire chromosomes are lost or gained, (ii) intrachromosomal instability, characterized by insertions, deletions, translocations, amplifications and other forms, all sharing the feature of utilizing DNA breakage as an early step. A relatively new technique known as inter-simple sequence repeat PCR (ISSR-PCR) has been shown to provide a simple and reliable measure of GI, particularly GI due to DNA breakage events (10–12). ISSR-PCR scans the genome for genetic alterations in an unsupervised manner, and would thus be expected to provide a 'global' measure of GI. A modified form of the technique known as fluorescent-ISSR-PCR has been shown to provide a 3-fold increase in product detection sensitivity (13–15), making it a potentially important tool for the study of GI in human cancer.

Initially isolated as the proto-oncogene pituitary tumor transforming gene (*PTTG*), securin is a key mitotic checkpoint protein involved at the metaphase–anaphase interface. Securin sequesters the active protease separase, preventing the premature separation of sister chromatids. Securin is overexpressed in pituitary, colon and other tumors (16–18). The first evidence of its role in the development of GI came from an *in vitro* study which demonstrated securin overexpression to induce aneuploidy, arising from chromatid missegregation (19). More recently we reported that securin can directly induce GI in thyroid cells (13).

Securin has been shown to be involved in both UV and chemotoxic DNA damage response pathways in mammalian cells (20,21). Expression of securin is downregulated both by DNA-damaging drugs such as doxorubicin and bleomycin, and by UV irradiation, prior to DNA repair. Theoretically, the markedly raised expression of securin apparent in many cancers would result in insufficient suppression of the protein, thereby interfering with DNA repair pathways which are mediated through securin, and resulting in the rapid accumulation of coding and non-coding changes in DNA sequence (21).

Securin has been shown to interact with DNA repair proteins, most notably p53 and Ku70 (22,23). Ku70 is involved in the protein kinase-dependent DNA (PK-DNA) double-strand break repair pathway, otherwise known as the non-homologous end-joining (NHEJ) pathway, the major mechanism employed by mammalian cells in repairing dsDNA breaks (22). In response to DNA damage, securin-Ku70 interaction has been shown to be inhibited, thus releasing free Ku70 to bind DNA at breakage sites, triggering the NHEJ repair mechanism (22). Dissociation of constitutive securin-Ku70 complexes may thus represent a critical process in the normal cellular response to DNA damage. However, one very recent report in colorectal cells has disputed a role for securin in GI (24). The exact implications of altered securin expression on DNA damage and its precise role in DNA repair therefore remain unresolved.

Based on a number of disparate *in vitro* investigations in human and yeast cells we hypothesized that securin induces GI in colorectal cells by interfering with DNA repair pathways, thus promoting transformation and tumorigenesis, and that this occurred via its reported interaction with Ku70. We therefore measured intrachromosomal GI in a cohort of colorectal tumors and examined associations between the degree of GI and the clinicopathological features of these tumors. We further investigated whether securin induces GI in human colorectal cancer cells. We demonstrate that securin has a causal role in the development of GI in colorectal cells *in vitro*, and that this process is due at least in part to the inhibition of Ku heterodimer, which is involved in the non-homologous end-joining DNA repair mechanism. We propose that GI induced by increased expression of securin represents a fundamental mechanism in colorectal tumorigenesis.

Materials and methods

Tumor specimens

Matched normal and tumor tissue specimens were obtained from 24 patients undergoing surgery for colorectal cancer at the University Hospital Birmingham NHS Trust, UK. Normal samples were obtained from adjacent colorectal tissue of non-tumorous appearance upon gross clinical inspection. These specimens were thereafter confirmed as normal or tumor samples by histopathological examination. All specimens, harvested at the time of resection, were snap frozen and stored at −80°C, and were collected with appropriate local ethical committee approval and informed patient consent. Full clinical and pathological data were recorded for each patient/specimen.

Cell lines, plasmids and transfections

Human colorectal cancer HCT116 cells (kindly provided by Prof. Vogelstein, MD, USA) were grown in McCoy's 5A modified medium, supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% pen/strep (Life Technologies, Inc., Grand Island, NY, USA). Non-transformed human fibroblasts (a kind gift from Dr Andrew Turnell, University of Birmingham, UK) were grown in Dulbecco's medium, supplemented with 8% fetal bovine serum and 10% sodium pyruvate.

Cells were transiently transfected using Fugene-6 reagent (Promega Corp., Madison, WI, USA), as per the manufacturer's instructions but with an optimized ratio of 3 µl per µg plasmid DNA. pCI-neo-*PTTG*, which housed the full-length human *PTTG*/securin cDNA, was kindly provided by Prof. Shlomo Melmed (University of California School of Medicine, Los Angeles, CA, USA). Control transfections used equal amounts of vector-only pCI-neo plasmid. For each well of a 6-well culture plate, 0.5 µg of plasmid was used for 'low dose' and 3 µg of plasmid was used for 'high dose' transfections. For all other transfection experiments, unless otherwise stated, 2 µg of plasmid DNA was used for each well of a 6-well plate. Cells were harvested in 1 ml Trizol Reagent, 48 h after transfection. Transfection efficiency was assessed via β-gal staining.

Securin knockdown experiments were carried out using pre-designed securin-specific siRNA (ID# 42068, Ambion Inc., Huntingdon, UK). A 'scrambled' siRNA (Negative control siRNA #1, ID# 4611, Ambion Inc., Huntingdon, UK) was used as negative control. HCT116 cells were transfected using siPORT NeoFX transfection reagent (Ambion Inc., Huntingdon, UK) as per manufacturer's instructions. In brief, cells were trypsinized and suspended in culture media and mixed with siRNA (10–30 nM) and NeoFX transfection reagent (3 µl ml^{-1} of medium) before plating and incubation for 48 h. Optimization studies demonstrated 30 nM siRNA transfections to provide optimal effect.

DNA and RNA extraction

Total RNA and DNA extraction from snap-frozen colorectal specimens and cell lines was performed with Trizol reagent (Sigma-Aldrich, Dorset, UK) (25,26). For DNA extraction, the interphase and organic phase of the extraction medium were precipitated with 100% ethanol, and the pellet washed twice with 0.1 M sodium citrate-ethanol, then re-pelleted and dried prior to dissolution in 8 mM NaOH solution. Total RNA and DNA were extracted from the same Trizol homogenate allowing mRNA expression and DNA analysis to be correlated.

Reverse transcriptase–PCR

RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA) in a total reaction volume of 20 µl, with 1 µg colon total RNA and 30 pmol random hexamer primers. Expression of specific mRNAs was determined using the ABI PRISM 7700 Sequence Detection System, as we have described previously (26). All reactions containing securin primers were multiplexed with a pre-optimized control probe and primer mix for 18S ribosomal RNA (PE Biosystems, Warrington, UK). Securin primer and probe sequences were as described previously (25). As per the manufacturer's guidelines, data were expressed as C_t values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔC_t values and, subsequently, fold changes in gene expression. All statistics were performed with ΔC_t values. Measurements were carried out a minimum of three times for each sample, and only specimens yielding consistent and repeatable expression data used in subsequent analyses. Reactions were 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min.

FISSR-PCR assay

FISSR-PCR amplifications were carried out using a 5′-FAM labeled primer $(CA)_8RG$ (R = a 50:50 mix of the purines adenine and guanine). As described previously, these primers are anchored at the 3′ ends of CA-repeat sequences found throughout the genome, and produce amplification of those DNA sequences, typically <2 kb in size, which are present between relatively close, inverted primer-binding repeat sequences (7,10). PCR reactions contained 5 ng of sample genomic DNA, 1× PCR reaction buffer (7.5 M Tris–HCl, pH 8.8, 2 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 1.5 mM $MgCl_2$, 1.5 µM primer and 0.2 mM mix of dNTPs, with 0.75 U of *Taq*DNA polymerase (Fermentas, Hanover, MD, USA). The thermal cycling conditions were as follows: initial denaturation of 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, final extension of 10 min at 72°C. One microliter of PCR product was mixed with 1.5 µl of 5× loading buffer (1:4 mixture of loading buffer and formamide; Sigma, Poole, UK), and 0.25 µl of GENESCAN-2500 ROX-labeled molecular weight standard (red fluorescence) (Applied Biosystems, Warrington, UK) was included in the loading samples. The samples were denatured at 95°C for 5 min before loading onto an ABI 377 automated sequencer (Applied Biosystems) and electrophoresed on 5% polyacrylamide gels under denaturing conditions containing 7 M urea in 1× TBE buffer. Three replicate experiments per sample were carried out, and specimens demonstrating consistent, repeatable data carried over into further analyses. Nineteen of the 24 samples fulfilled the stringency of this criterion.

For *ex vivo* tissue analysis, we determined the degree of GI according to Basik *et al*. (10,11), by dividing the number of altered bands in the tumor by the total number of bands in the corresponding normal tissue sample (multiplied by 100 to form a percentage) to generate the GI index (GI index). For cell line experiments, the number of altered bands in the experimental/treated cells was divided by the total number of bands in the untreated control cells harvested at the same timepoint (48 h, unless specified). The GI index represents the standard measure of GI with ISSR-PCR analysis.

Western blot analysis

Whole cell protein extracts were prepared from colon tissues in lysis buffer [100 mM sodium chloride, 0.1% Triton X-100 and 50 mM Tris (pH 8.3)] containing enzyme inhibitors. Nuclear and cytosolic protein subfractions were prepared using a commercial Nuclear extraction kit (Active Motif Europe, Rixenhart, Belgium) as per manufacturer's instructions. Protein concentration before loading was measured by the Bradford assay with BSA as standard.

Western blot analyses were performed as we have described previously (25,26). Briefly, soluble proteins (30 µg) were separated by electrophoresis in 12.5% sodium dodedecyl sulfate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5% non-fat milk in PBS with 0.1% Tween, followed by incubation with a monoclonal securin antibody (1:1000 concentration) (NeoMarker, Fremont, CA, USA) or a polyclonal Ku70 antibody (1:500) (Santa Cruz Biotech., Santa Cruz, CA, USA). After washing in PBS plus 0.1% Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. After additional washes, antigen–antibody complexes were visualized by the ECL chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK). α-Tubulin (Santa Cruz, CA, USA; used at a concentration of 1:1000) and Lamin (Santa Cruz, USA; used at a concentration of 1:500) were used to determine purity and equal loading of cytosol and nuclear protein subfractions respectively. β-Actin expression (monoclonal anti-β actin clone AC-15, used at 1:10 000; Sigma-Aldrich, Poole, UK) was also determined to control for differences in protein loading between

different groups. Scanning densitometric analysis of western blotting assessment of securin expression in duplicate experiments was normalized to β-actin.

Apoptosis assay: caspase 3 and 7 activity

The effect of increased securin expression and etoposide on caspase activity was determined using the Caspase-Glo assay (Promega Corp. Madison, WI, USA). An aliquot of 2×10^4 cells per well were incubated in white 96-well plates (Corning corp., USA). As a positive control for apoptosis, 24 h after transfection the chemotoxic agent etoposide (4 μM) was added to the cell medium. Caspase activity was measured 48 h after transfection. Fluorescence was detected 90 min after addition of the homogenous caspase 3/7 reagent at an excitation wavelength of 485 nm and emission wavelength of 520 nm using an Orion Luminometer (Flowgen, UK). All samples were assayed in triplicate.

Host cell reactivation assays

The reporter plasmid pGL2bluc (Promega Corp. Madison, WI, USA) containing the firefly luciferase gene (driven by the CMV promoter) was treated for 5 h at 37°C with 4 μM etoposide in a cleavage reaction mixture containing 50 mM Tris–HCl buffer pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM EDTA, 30 μg ml^{-1} BSA and 10 U of human topoisomerase IIα (Sigma-Aldrich, Dorset, UK). Damaged plasmid DNA was recovered by standard ethanol precipitation. Cells seeded in 24-well plates were transiently transfected with securin or vector-only for 24 h. Thereafter, 0.5 μg of etoposide-treated reporter plasmid and 0.25 μg of control plasmid (pRL-CMV) were mixed with Fugene-6 transfection agent prior to addition to the HCT116 cells and incubation for 48 h. The luciferase activity of cell lysates was measured using a dual luciferase reporter assay system (Promega Corp., Madison, WI, USA). Briefly, 20 μl of lysate was mixed first with 100 μl of luciferase assay reagent (LAR II) and then 100 μl of Stop & Glo reagent. Relative light units (RLU) were measured immediately after addition of each reagent using an Orion Luminometer (Flowgen, UK). For each lysate sample, the light intensity for the firefly luciferase activity was then adjusted relative to the control renilla luciferase activity.

Ku70-DNA binding assay

Nuclear and cytosolic protein extracts were prepared using the commercial Nuclear Extraction kit (Active Motif Europe, Rixenhart, Belgium). Ku70 activation was measured using an ELISA based Ku70-DNA binding assay (Active Motif, Belgium). This is a quantitative functional assay that measures Ku70 DNA binding capacity, and consists of linear oligonucleotides with blunt ends immobilized onto assay plates, with binding of Ku70 protein determined through a primary antibody to an epitope of Ku70 only available upon DNA binding. A secondary HRP-conjugated antibody was used for colorimetric quantification of Ku70 activation in protein lysates, and was prepared as per manufacturer's instructions. Further, a Ku70 competitor oligonucleotide was utilized to monitor the specificity of the assays.

Statistical analyses

Statistical analyses were performed using Minitab version 14 software. Student's *t*-test and the Mann–Whitney test were used for comparison between two groups of parametric and non-parametric data, respectively. Significance was taken as $P < 0.05$.

Results

GI and securin expression in colorectal tissues and HCT116 cells

Nineteen of our 24 patient-matched colorectal normal and tumor tissues yielded consistent and reproducible FISSR-PCR data. GI index values ranged from 2.04 to 46.72% (mean 21.27%; median 20.09%) (Figure 1A). When we investigated whether the degree of GI measured in colorectal cancers by FISSR-PCR was associated with Dukes' staging, we observed greater GI values for Dukes' stage C&D tumors (GI index 27.41% ± 4.18, $n = 10$) compared with Dukes' A&B tumors (13.46% ± 3.22, $n = 9$; $P = 0.024$) (Figure 1A).

In corroboration of an earlier finding (17), securin mRNA expression was higher in colon cancers compared with matched normal tissues (3.12-fold, $n = 22$, $P = 0.007$; Figure 1B), with protein demonstrating parallel changes, and was higher in Dukes' stage C&D colorectal tumors (4.42 ± 1.51-fold, $n = 12$) than in Dukes' stage A&B tumors (2.71 ± 0.89-fold, $n = 9$, $P = 0.033$). An example of the FISSR-PCR banding pattern in a representative normal and matched tumor specimen is provided in Figure 1B.

Having characterized the GI and securin expression profiles of our cohort, we next investigated whether securin could induce GI in human colorectal HCT116 cells *in vitro*. In comparison to both untransfected and vector-only-transfected controls, HCT116 cells overexpressing securin exhibited a dose-dependent increase in GI [GI index vector-only (VO)-control = 11.65 ± 0.46% versus GI index 'low dose' securin = 18.23 ± 2.08%, $n = 4$; $P = 0.01$. GI index 'High dose' securin = 46.13 ± 3.68%, $n = 4$, $P < 0.001$] (Figure 1C). There was no significant difference in GI between untransfected cells (UT) and VO-control HCT116 cells.

Securin also elicits GI in non-transformed fibroblasts, but does not induce apoptosis

Given that HCT116 cells constitutively express high securin levels, we performed parallel transfection studies in non-tumorous human fibroblasts. These are cells which are relatively genetically stable, with comparatively low expression of securin (Figure 2A). Consistent with the findings for HCT116 cells, we observed a significant increase in GI, in a dose-dependent manner, in fibroblasts transfected with securin compared with VO-control cells (GI index VO-control = 7.0 ± 1.34% versus GI index 'low dose' securin = 13.03 ± 0.72%, $n = 4$; $P = 0.008$. GI index 'High dose' securin = 17.0% ± 1.17, $n = 4$, $P = 0.002$) (Figure 2A).

To investigate under-expression of securin in HCT116 cells, we performed transient knockdown of securin. We elected not to use $HCT116^{securin-/-}$ cells in this context, given that they are inherently unstable (27). Specific siRNA knockdown of securin expression in native HCT116 cells was associated with a significant reduction in the degree of endogenous GI compared with control cells transfected with 30 nM 'scrambled' negative control siRNA (securin-siRNA GI index = 12.6 ± 0.35%; scrambled siRNA GI index = 7.93 ± 0.67%, $n = 4$; $P < 0.001$) (Figure 2B). After 48 h, there was no significant difference in GI between untreated cells and scrambled siRNA-transfected negative control HCT116 cells.

To investigate the possibility that the FISSR-PCR GI measurements in HCT116 cells reflected detection of DNA degradation associated with apoptosis, we investigated whether significant apoptosis was induced during the *in vitro* experiments above. Caspase 3 and 7 enzyme activity in securin-transfected HCT116 cells was compared with both untransfected (negative control) and VO-control-transfected cells (Figure 2C). There was no increase in caspase 3/7 activity in securin overexpressing cells compared with VO-transfected cells (VO = 0.89 ± 0.04 RLU (relative light units)/RLU untransfected cells (control); securin = 0.98 ± 0.05 RLU/RLU control; $n = 9$; P = NS). In contrast, the positive control of etoposide treatment induced a significant increase in caspase activity (etoposide = 1.94 ± 0.16 RLU/RLU control; $P < 0.001$; $n = 9$).

Taken together, these data suggest that modulation of securin expression is involved in inducing GI in transformed HCT116 cells as well as in non-tumorous human fibroblasts,

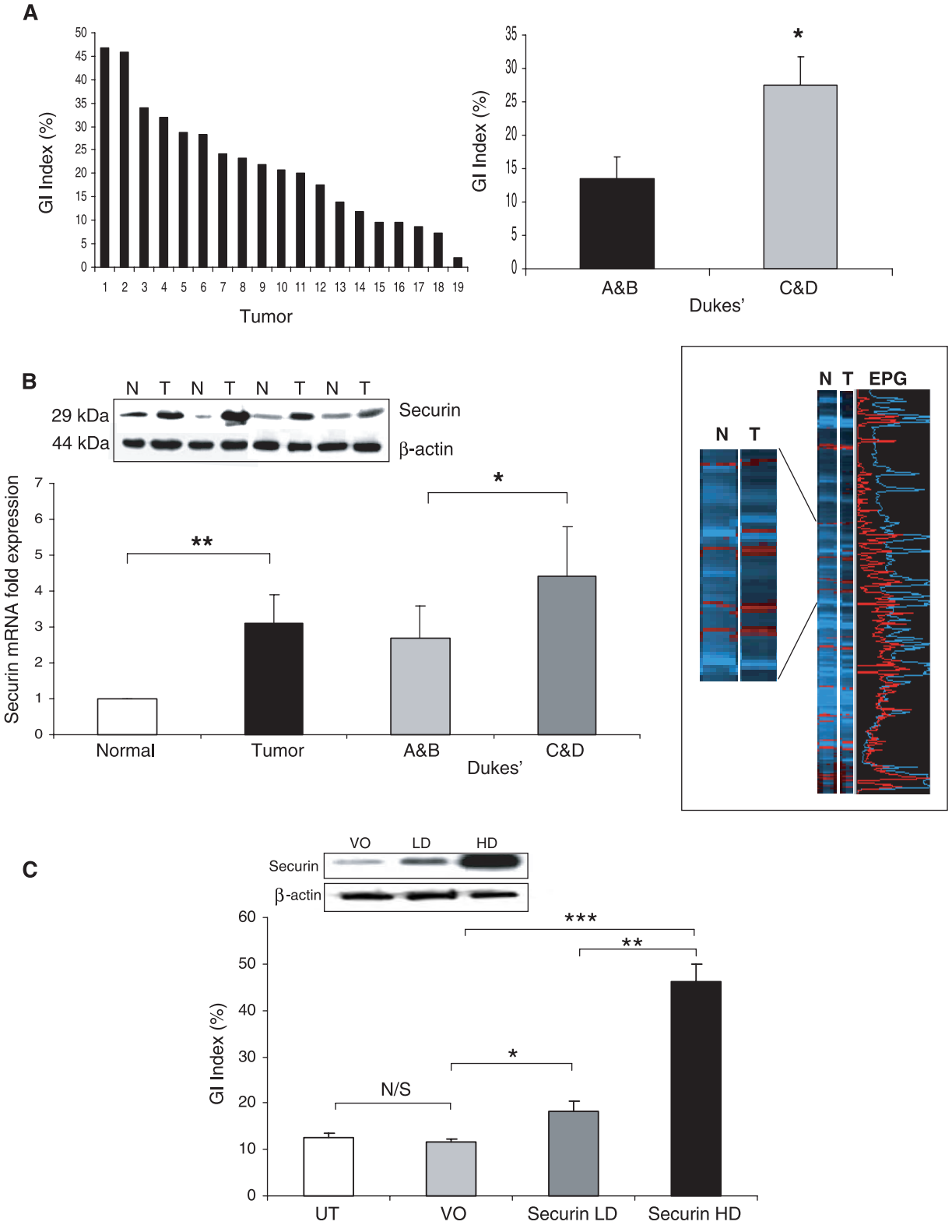

Fig. 1. Securin expression and genetic instability in colorectal cancers and HCT116 cells. (**A**) Genetic instability index (GI index) values for 19 colorectal cancers compared with subject-matched normal tissue (obtained from the same patient to that of the cancer) plotted in order of decreasing GI index value, and mean GI index for Dukes' stage C&D tumors compared with Dukes' stage A&B tumors. (**B**) Securin mRNA expression in colorectal cancers compared with patient-matched normal tissues, with subdivision into Dukes' stage C&D and stage A&B tumors. In the 22 samples providing consistent gene amplification, securin mRNA expression is plotted as mean fold difference in securin expression in cancers compared with patient-matched normal. Upper panel: Representative western blotting of securin protein expression in colorectal tumors (T) compared with patient-matched normal tissues (N). Right panel: a representative example of FISSR-PCR banding in a normal (N) and matched tumor (T), with electropherogram (EPG) output, used to calculate band size and intensity changes. (**C**) GI index values for HCT116 cells transfected with 'high dose' (*HD*; 3 μg DNA per well of a 6-well plate) and 'low dose' (*LD*; 0.5 μg DNA per well) securin for 48 h, versus untransfected (UT) and vector-only (VO) control cells. Upper panel: Western blot demonstrating increased expression of securin in LD and HD-transfected HCT116 cells compared with VO-control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

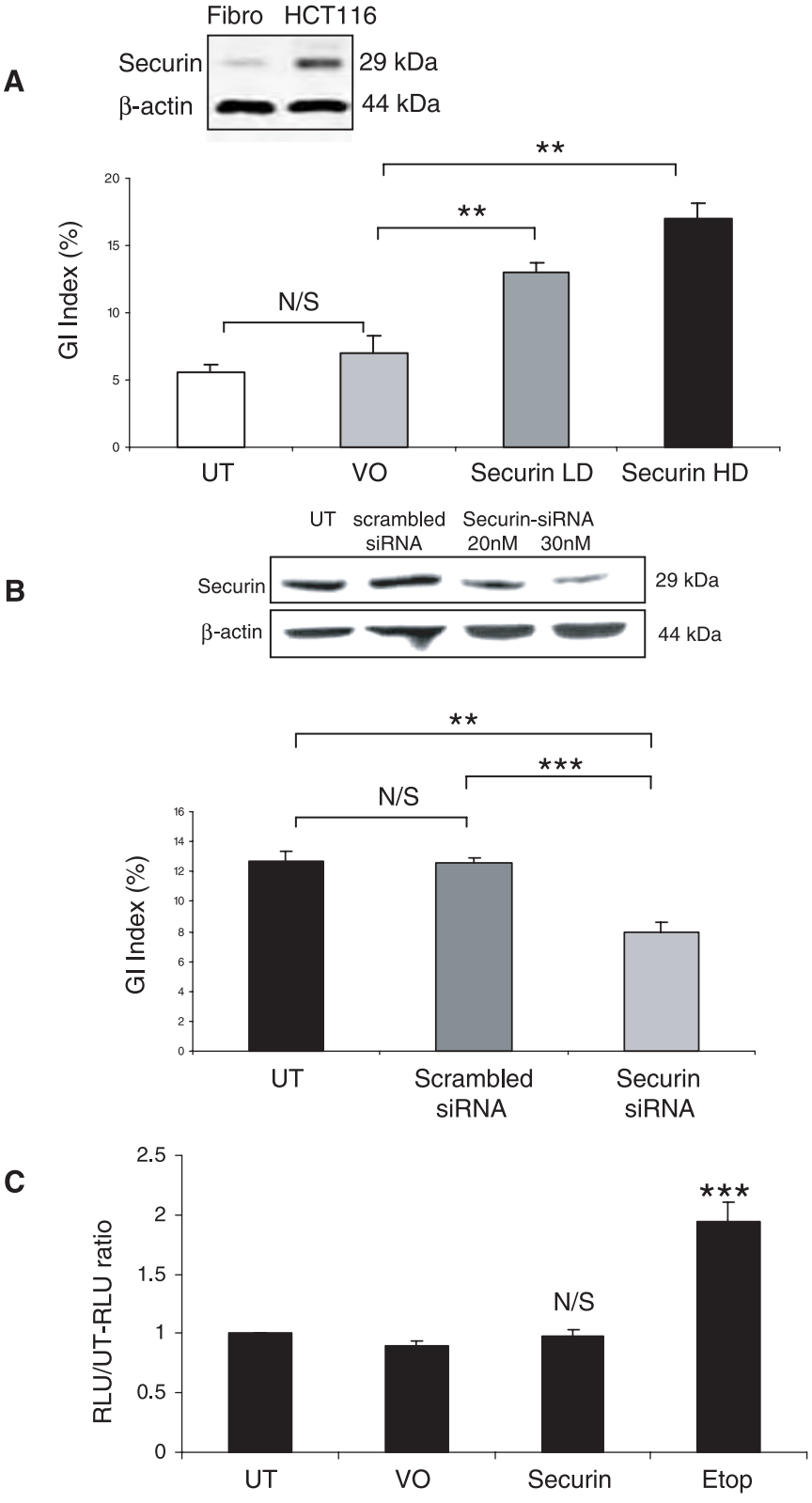

Fig. 2. Securin elicits GI in non-transformed fibroblasts, but does not induce apoptosis. (**A**) Dose-dependent induction of genetic instability in human fibroblasts by securin. Upper panel: Endogenous expression of securin protein is lower in human fibroblasts than transformed HCT116 cells, demonstrated by western blotting. Lower panel: Cells were transfected with 'high dose (HD)' and 'low dose (LD)' securin for 48 h (LD = low dose securin (0.5 μg DNA per well of 6-well plate); HD = high dose securin (3 μg DNA per well)). (**B**) Upper panel: Knockdown of securin expression using specific siRNA (20–30 nM), in comparison to cells transfected with 30 nM scrambled siRNA as negative control. *Below* GI index values for HCT116 cells transfected with 30 nM securin-specific siRNA versus untransfected cells (UT) harvested at the same timepoint, and negative-control cells transfected with scrambled siRNA. (**C**) Functional caspase 3 and 7 apoptosis assays in HCT116 cells transfected with securin and empty plasmid (VO) and harvested after 48 h. Caspase activity was similar in securin-transfected and VO-control cells. Luciferase activity representing caspase function is expressed as a ratio of the relative light unit (RLU) measured to that of untransfected (UT) cells. (N/S = non-significant. VO = vector-only control; Etop = etoposide, treated as a positive control). $^{**}P < 0.01$, $^{***}P < 0.001$.

and that the detection of GI is unlikely to reflect significant securin-mediated apoptosis.

Securin suppresses DNA repair activity in HCT116 cells by repressing Ku70 activity

To examine whether securin's induction of GI reflects a suppression of DNA repair in human colorectal cells, we performed dual-luciferase reporter based Host-Cell Reactivation Assays. Compared with VO-control cells, etoposide-induced dsDNA damage repair activity was significantly diminished in HCT116 cells transfected with securin (VO 64.0 ± 2.0 RLU versus Securin 51.4 ± 1.7 RLU; $n = 6$; $P < 0.001$) (Figure 3Ai). We also repeated these experiments in our non-tumorous human fibroblasts (Figure 3Aii). Here, on a background of low endogenous securin expression, the effect was even more marked, with dsDNA damage repair activity diminished from 50.3 ± 3.0 RLU in VO-transfected cells compared with 12.8 ± 0.6 RLU in securin-transfected cells ($n = 6$; $P < 0.001$).

When we examined native Ku heterodimer activity using a Ku70 DNA-binding assay, we observed that HCT116 cells transfected with securin demonstrated significantly lower Ku70 functional capacity compared with VO-control cells, both in nuclear and whole cell extracts (nuclear extract: VO-control = 0.30 ± 0.01 AU versus securin = 0.25 ± 0.01, $n = 4$, $P = 0.002$; whole cell: VO-control = 0.44 ± 0.02 AU versus securin = 0.36 ± 0.01, $n = 4$, $P = 0.006$) (Figure 3B).

Whole cell Ku70 function was significantly greater in HCT116 cells exposed to 4 μM etoposide compared with untreated cells (Figure 3C). This was observed for both VO-control (VO-control = 0.44 ± 0.02 AU versus VO + etoposide = 0.51 ± 0.01, $n = 3$, $P = 0.02$) and securin-transfected cells (securin = 0.36 ± 0.01 versus securin + etoposide = 0.45 ± 0.02 AU, $n = 3$, $P < 0.001$) (Figure 3C). Therefore, although total Ku70 DNA-binding activity is higher in response to dsDNA damage, securin represses Ku70 DNA-binding activity in HCT116 cells, both in the presence and absence of DNA-damaging etoposide.

Securin protein expression, cellular localization and response to DNA damage

Western blotting analysis demonstrated endogenous securin protein expression in HCT116 cells to be predominantly nuclear, with significantly less expression in the cytosol (Figure 4A). This expression pattern was also maintained in cells transfected with exogenous securin. However, in response to etoposide-induced DNA damage, we observed a marked reduction in securin expression both in cells transfected with empty vector or with securin (Figure 4B).

Western blotting analysis performed using nuclear and cytosolic protein extracts demonstrated that the observed reduction in securin expression occurred predominantly in the nucleus (Figure 4C). In addition, in response to 6 and 24 h exposure to etoposide, there was a clear reversal in the cellular pattern of securin expression in both untransfected and securin-transfected cells (Figure 4C), in that securin expression became higher in the cytoplasm than the nucleus. However, HCT116 cells exposed to etoposide for only 6 h and then allowed to recover in fresh media for 18 h demonstrated a pattern of cellular expression of securin similar to untreated cells, with predominant expression in the nucleus. These findings were also observed in HCT116 cells transfected with exogenous securin (Figure 4D), suggesting both an overall reduction in securin expression and an alteration in its nuclear:cytoplasmic balance in response to etoposide-induced DNA damage.

Ku70 expression and cellular localization in response to DNA damage

In common with our Ku70 activity data, western blotting analysis demonstrated Ku70 protein to be expressed in both nuclear and cytosolic compartments of HCT116 cells (Figure 5A). However, there was no change in the total level of Ku70 protein and cell expression pattern in cells transfected with securin. We also examined total Ku70 protein expression in non-transformed diploid fibroblast cells, in response to securin overexpression. In common with HCT116 cells, no overall change in whole cell Ku70 expression occurred following transient overexpression of securin (Figure 5B).

In response to etoposide-induced DNA damage, there was no significant alteration in total Ku70 expression in HCT116 cells (Figure 5C). In contrast, western blotting analysis demonstrated a clear change in the subcellular localization of Ku70 expression in response to etoposide exposure (Figure 5D). Greater Ku70 protein expression was observed in the nucleus compared with the cytosolic compartment of HCT116 cells after 24 h of etoposide exposure both in the presence and absence of securin. Taken together, these data suggest that translocation of cytosolic Ku70 protein into the nuclear compartment is elicited by etoposide-induced DNA damage, but not by securin overexpression.

Discussion

GI in colorectal cancer is associated with metachronous tumors, the development of recurrence and metastasis, and poor survival (2,8,9). By comparing GI and known patient/tumor clinicopathological data, we observed significantly higher GI index values for Dukes' stage C&D tumors compared with stage A&B tumors, and confirmed a previous study which reported the highest securin expression in advanced colorectal tumors (17). GI is essentially cumulative, and larger tumors would inevitably represent more cell divisions than smaller ones. An alternative explanation of our data is that GI differences between the Dukes' stage classifications reflect potential differences in tumor volume, rather than merely levels of securin expression. As we have no data on the rate of tumor growth, or on tumor volume, the current investigation is not able to rule out this possibility.

Recently, it has been proposed from a study in human colorectal HCT116$^{securin-/-}$ cells that securin is not critical for chromosomal stability (24). In our hands, and in common with the original description of these cells (27), HCT116$^{securin-/-}$ cells are catastrophically unstable, and unsuitable for long-term experiments. Furthermore, *Securin*$^{-/-}$ mouse embryo fibroblasts exhibit aberrant cell cycle progression with prolonged G_2/M phase, chromosome breaks and premature centromere division (28). To differentiate between these two opposing sets of observations, we therefore examined the relationship between securin expression and GI in parental HCT116 cells overexpressing and underexpressing securin. We observed a dose-dependent increase in GI in response to securin overexpression. Given that securin-null mice are viable, *securin* deletion may elicit mechanisms of mitotic compensation, enabling cells to still

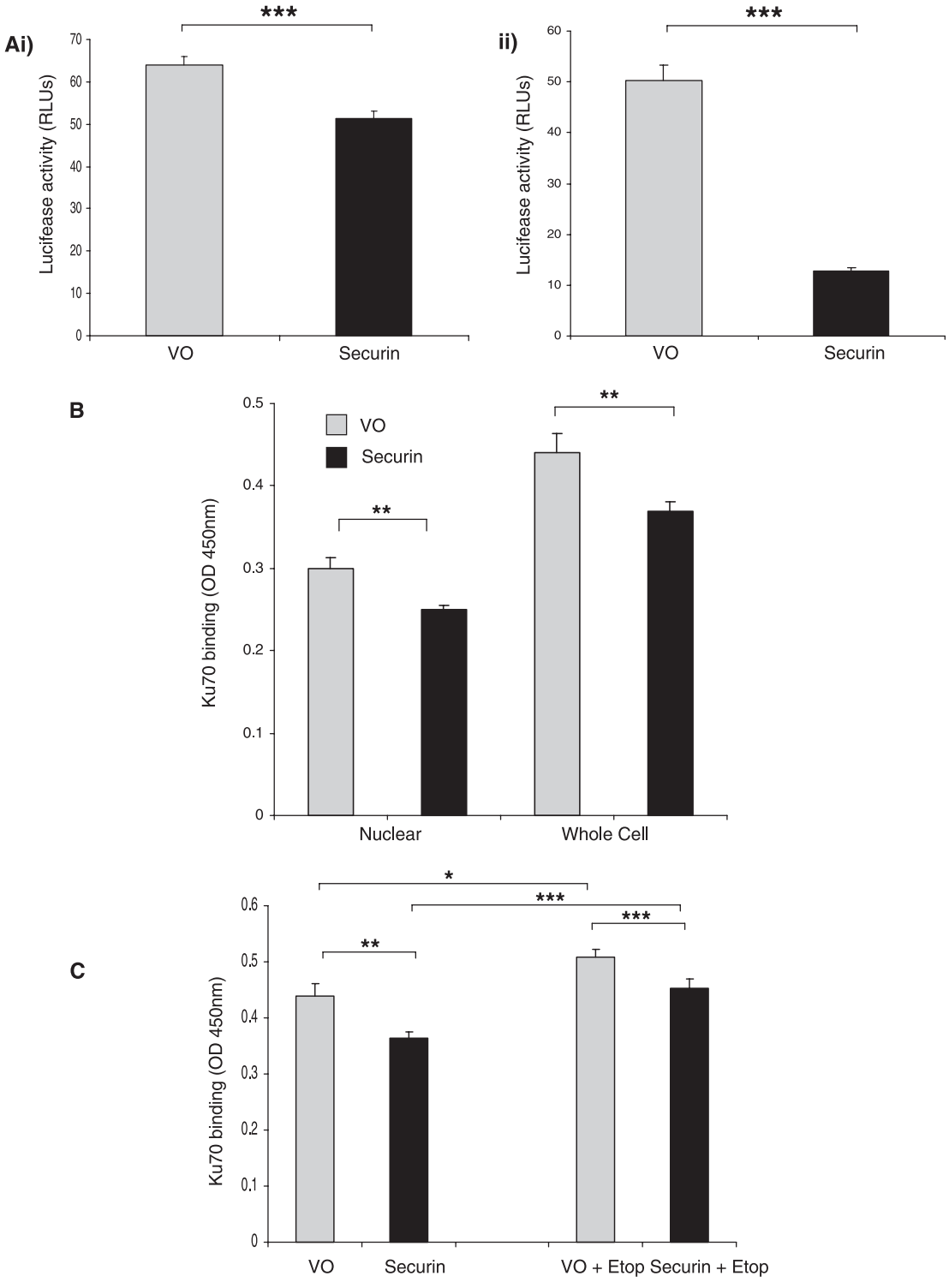

Fig. 3. Securin inhibits double-stranded DNA repair activity in HCT116 cells by inhibiting Ku70 DNA-binding activity. (**A**) Host Cell Reactivation assay, where luciferase activity is proportional to the amount of repair of the pre-damaged luciferase gene. (i) HCT116 cells overexpressing securin were associated with significantly reduced luciferase activity, signifying less repair activity compared with VO-control. (ii) Host cell reactivation assays in non-transformed diploid fibroblasts, demonstrating significantly lower repair activity of cells transiently transfected with securin than vector-only. (**B**) Ku70 DNA binding assay in nuclear and whole cell lysates from securin-transfected HCT116 cells compared with activity in VO-transfected control cells. **C**. KU70 function in whole cell lysates. Activity in untreated HCT116 cells transfected with VO and securin constructs, compared with cells exposed to 4 μM etoposide (24 h) and transfected with VO and securin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

divide, albeit with a raised frequency of missegregation (27). However, our study and others (13,19,29) strongly suggest that when securin is present, 'too much' or 'too little' expression of the gene promotes GI *in vitro*.

As HCT116 cells express high constitutive securin levels, it is possible that further increases in its expression in these cells may provide artifactual effects. We therefore performed parallel experiments in non-tumorous human fibroblasts

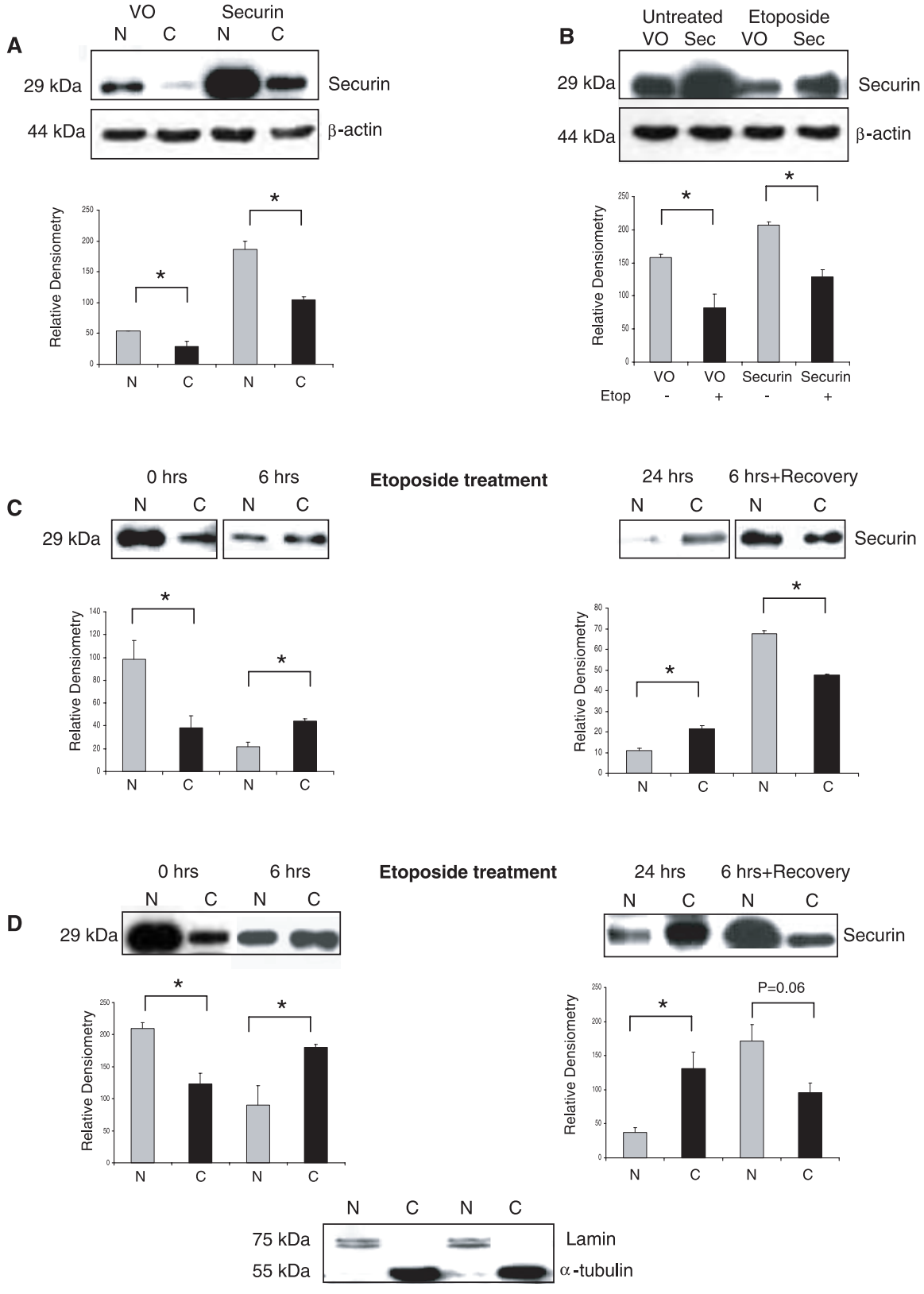

Fig. 4. Securin expression and localization HCT116 cells. (**A**) Securin expression and cell localization demonstrated using separate nuclear (N) and cytosolic (C) extracts from securin and vector-only (VO)-transfected HCT116 cells. β-Actin was used to demonstrate total protein loading. Scanning densitometry from two separate experiments is tabulated below each representative western blot. *$P < 0.05$. (**B**) Change in total securin expression in response to etoposide exposure (24 h) in HCT116 cells. Lower panel: scanning densitometry comparing the effect of etoposide treatment on total securin expression in VO (first two bars) and securin (right-hand bars)-transfected cells. (**C**) Change in cell localization of endogenous securin protein in vector-only-transfected HCT116 cells in response to 6 and 24 h etoposide treatment in nuclear and cytosolic protein extracts, with the re-constitution of native securin protein localization in cells transiently exposed to etoposide (6 h) and bathed in fresh medium for 18 h. (**D**) Change in cell localization of securin protein in securing-transfected HCT116 cells. Lower panel: Representative western blotting detection of α-tubulin and lamin, demonstrating purity of the nuclear–cytoplasmic separation.

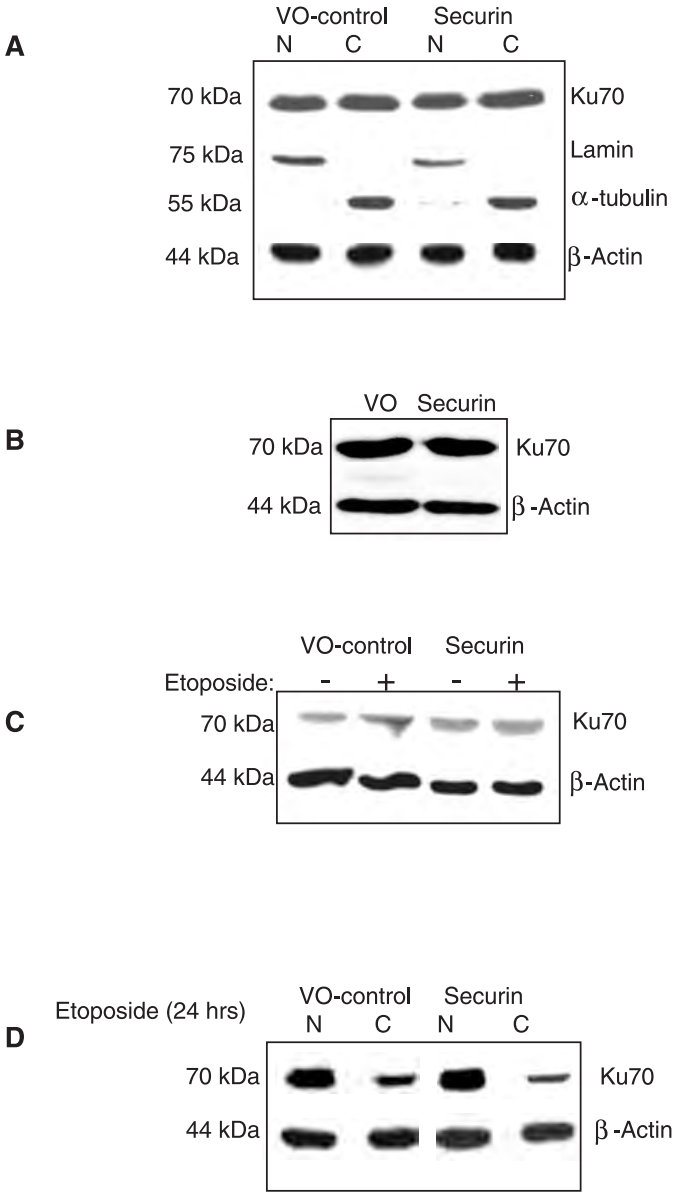

Fig. 5. Ku70 expression and localization in HCT116 cells. (**A**) KU70 expression and cell localization demonstrated using separate nuclear (N) and cytosolic (C) extracts from securin and vector-only (VO)-transfected HCT116 cells. Lamin and α-tubulin was used to demonstrate clean separation of cytosolic and nuclear proteins. β-Actin was used to assess protein loading. (**B**) Ku70 protein expression in untransformed diploid fibroblasts transiently transfected with securin and vector-only. (**C**) Ku70 expression in HCT116 cells treated with and without etoposide for 24 h. (**D**) Change in Ku70 cell localization in response to etoposide treatment (24 h), in the presence and absence of exogenous securin.

which express low securin levels. Consistent with HCT116 cell data, a dose-dependent increase in GI was observed in securin-transfected fibroblasts compared with control cells.

One report has suggested increased securin expression to promote cell apoptosis (30). It is feasible that false positive results may result from the detection of apoptotically degraded DNA fragments by the sensitive FISSR-PCR method. However, we showed no significant increase in caspase 3 and 7 activity in HCT116 cells highly expressing securin compared with vector-only control cells, suggesting that the FISSR GI values accurately represent DNA sequence changes rather than apoptotic DNA fragmentation.

The precise mechanism involved in the observed securin-driven GI is not known. Securin has been shown to interact with Ku heterodimer which plays a critical role in repairing double-stranded DNA (dsDNA) breaks (22). More recently, securin's interaction with separase, and separase's subsequent stabilization, was proposed as a novel and potentially important DNA DSB repair mechanism in fission yeast (31). Therefore, we investigated whether elevated securin expression may inhibit repair of dsDNA breaks in human cells. We demonstrated securin overexpression to significantly inhibit dsDNA repair activity in non-transformed human fibroblasts and HCT116 cells.

As securin interacts with Ku70 (22), we sought to test whether high securin expression in HCT116 colorectal cells suppressed Ku70 DNA-binding function. We employed a quantitative functional assay that detects binding of Ku70 protein in cell lysates to DNA free-ends *in vitro*. The DNA end-binding capacity of Ku70 was significantly reduced in HCT116 cells overexpressing securin protein. Detection and binding to free DNA ends exposed as a result of dsDNA breaks by Ku70 is the first critical step in the NHEJ repair pathway. Repression of this major DNA repair mechanism may thus be important in the observed induction of GI by elevated securin expression in colorectal cells.

As a mitotic checkpoint protein, securin binds to and inhibits separase function, thereby preventing premature sister chromatid separation during mitosis (32). It has been proposed that securin may similarly function to constitutively sequester and inhibit Ku70 binding to exposed DNA ends (22). To investigate this hypothesis further, we examined securin and Ku70 protein expression and cellular localization. Ku70 protein expression was relatively evenly distributed between the nucleus and the cytosol of HCT116 cells. Consistent with observations made previously in JEG-3 placental cells (33), we showed that the majority of securin protein was expressed within the nucleus in HCT116 cells. It is therefore likely that within the nucleus there exists a high securin:Ku70 protein concentration ratio, whereas within the cytosol there is a low securin:Ku70 ratio. We also demonstrated an increase in total Ku70 function in response to DNA damage. Given that securin expression is reduced whereas Ku70 protein expression is unaltered in response to DNA damage, the observed increase in whole cell Ku70 activity would be anticipated. These observations lend further support for the hypothesis that securin constitutively interacts with and inhibits Ku70 function in human cells.

In response to DNA damage, Ku70 has been shown previously to translocate into the nuclear compartment from the cytosol (34), and there is growing evidence that movement of the protein is a critical regulatory mechanism for Ku70 function (35,36). In support, we also demonstrated translocation of Ku70 protein into the nucleus in response to etoposide insult. In contrast to the Ku70 nuclear shift, we observed that securin protein demonstrated the opposite change in subcellular distribution. In response to DNA damage, higher securin concentration was apparent within the cytosol of HCT116 cells. The reversal of the cellular distribution of securin is a novel finding which sheds further light on its undoubtedly complex role in DNA damage. Of pertinence is the implication that reduced nuclear expression of securin, coupled with increased Ku70 availability, would be predicted to strongly augment the Ku70-mediated DNA

damage response. However, further studies are necessary to clarify this hypothesis.

In summary, we have demonstrated that GI in Dukes' C&D tumors was significantly higher than in Dukes' stage A&B tumors. Measurement of GI using FISSR-PCR may therefore represent a useful prognostic marker in colorectal cancer. *In vitro* experiments revealed a causal role for securin overexpression in generating GI in colorectal cancer cells, most likely through the inhibition of DNA repair activity. Although other genes and pathways may well be involved, inhibition of Ku70 function by altered cellular distribution of securin is likely to be a critical mechanism in DNA damage, given the importance of Ku70 in the non-homologous end-joining DNA repair pathway. We therefore propose that *securin* is pivotal to the development of GI in colorectal cells and their subsequent tumorigenesis and progression.

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