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Prevalence and Functional Analysis of Sequence Variants in the ATR Checkpoint Mediator Claspin

Jianmin Zhang,[1] Young-Han Song,[1] Brian W. Brannigan,[1] Doke C.R. Wahrer,[1] Taryn A. Schiripo,[1] Patricia L. Harris,[1] Sara M. Haserlat,[1] Lindsey E. Ulkus,[1] Kristen M. Shannon,[1] Judy E. Garber,[2] Matthew L. Freedman,[3] Brian E. Henderson,[4] Lee Zou,[1] Dennis C. Sgroi,[1] Daniel A. Haber,[1] and Daphne W. Bell[1]

[1]Massachusetts General Hospital Cancer Center and Harvard Medical School, Charlestown, Massachusetts; [2]Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts; [3]Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, and Broad Institute for Biomedical Research, Boston, Massachusetts; and [4]Department of Preventive Medicine, University of Southern California Keck School of Medicine, Los Angeles, California

Abstract

Mutational inactivation of genes controlling the DNA-damage response contributes to cancer susceptibility within families and within the general population as well as to sporadic tumorigenesis. *Claspin* (*CLSPN*) encodes a recently recognized mediator protein essential for the ATR and CHK1-dependent checkpoint elicited by replicative stress or the presence of ssDNA. Here, we describe a study to determine whether mutational disruption of *CLSPN* contributes to cancer susceptibility and sporadic tumorigenesis. We resequenced *CLSPN* from the germline of selected cancer families with a history of breast cancer ($n = 25$) or a multicancer phenotype ($n = 46$) as well as from a panel of sporadic cancer cell lines ($n = 52$) derived from a variety of tumor types. Eight nonsynonymous variants, including a recurrent mutation, were identified from the germline of two cancer-prone individuals and five cancer cell lines of breast, ovarian, and hematopoietic origin. None of the variants was present within population controls. In contrast, mutations were rare within genes encoding the CLSPN-interacting protein ATR and its binding partner ATRIP. One variant of CLSPN, encoding the I783S missense mutation, was defective in its ability to mediate CHK1 phosphorylation following DNA damage and was unable to rescue sensitivity to replicative stress in CLSPN-depleted cells. Taken together, these observations raise the possibility that *CLSPN* may encode a component of the DNA-damage response pathway that is targeted by mutations in human cancers, suggesting the need for larger population-based studies to investigate whether *CLSPN* variants contribute to cancer susceptibility. (Mol Cancer Res 2009;7(9):1510–6)

Introduction

Disruption of the DNA-damage response pathway plays a critical role in the development of both sporadic and inherited forms of breast cancer and other cancers (1). For example, somatic mutations within *p53*, a key effector of the cellular response to DNA damage, are present in more than half of all sporadic tumors, whereas germline *p53* mutations are linked to Li-Fraumeni syndrome, a multicancer phenotype that includes breast cancer (2, 3). Germline mutations in *BRCA1* and *BRCA2*, genes which are also implicated in the cellular response to DNA damage, are responsible for a subset of familial breast cancer (4, 5), whereas lower-penetrance alleles in the *CHEK2, PALB2*, and *BACH1/BRIP* DNA-damage response genes are associated with a modest increase in the risk of developing this disease (6-10).

In mammalian cells, stalling of DNA replication forks, repair of UV-induced DNA damage, and exonuclease-mediated processing of double-strand DNA breaks all lead to the appearance of ssDNA and, consequently, to the induction of cell cycle arrest via a complex signal transduction cascade (1). An early event in the DNA-damage response to ssDNA is the recruitment of the ataxia telangiectasia and Rad3-related (ATR) protein, together with its binding partner ATRIP, to sites of DNA damage (11, 12). Once activated, ATR can phosphorylate the CHK1 kinase on Ser^{317} and Ser^{345} (13, 14). Activated CHK1, in turn, phosphorylates the CDC25A and CDC25C phosphatases, leading to ubiquitin-mediated proteosomal degradation of CDC25A and nuclear export and inactivation of CDC25C (15, 16). Consequently, CDKs remain in an inactive, phosphorylated state thus preventing cell cycle progression.

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Current address for D.W. Bell: Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD 20892.
Current address for Y-H. Song: Ilsong Institute of Life Science, Hallym University, Anyang 431-060, Korea.
Current address for M.L. Freedman: Dana-Farber Cancer Institute, Boston, MA 02115 and Broad Institute of Harvard and MIT, Cambridge, MA 02141.
Requests for reprints: Daniel Haber, MGH Cancer Center, Building 149, CNY7, 13th Street, Charlestown, MA 02129. Phone: 617-726-5628; Fax: 617-726-5637. E-mail: Haber@helix.mgh.harvard.edu

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In *Xenopus*, the activation of chk1 by atr following replicative stress is dependent on Claspin, a so-called mediator protein that, in a phosphorylated state, binds to chk1 (17-19). In mammalian cells, the orthologous protein, CLSPN, also mediates the activation of CHK1 following replicative stress or DNA damage (20, 21). This activity is dependent on the phosphorylation of CLSPN at Thr916, a requirement for binding of CLSPN to CHK1 *in vivo* (22). Consistent with its role in the DNA-damage response, small interfering RNA (siRNA)–mediated down-regulation of CLSPN in mammalian cells leads to an increase in premature chromatid condensation following hydroxyurea treatment, a reduction in the inhibition of DNA synthesis following UV exposure, and a decrease in cell survival (20). Recent studies have shown that CLSPN specifically accumulates during the S phase of the cell cycle, and that its degradation in the G_2 phase is essential for checkpoint recovery and associated entry into mitosis (23-26). Of note, Mrc1, the yeast homologue of CLSPN, is a component of normal DNA replication forks and has checkpoint-independent functions (27-31). Human CLSPN has recently been shown to facilitate the ubiquitination of a proliferating cell nuclear antigen following DNA damage, independently of ATR (32).

Here, we describe the resequencing of *CLSPN*, a relatively poorly characterized component of the DNA-damage response pathway, from the germ line of familial cancer cases and within a panel of sporadic cancer cell lines. We report prevalent variants within *CLSPN* as well as a single allelic variant within *ATRIP*. Functional analysis revealed that at least one variant of *CLSPN* is associated with hypersensitivity to replication-induced DNA damage both *in vitro* and *in vivo*.

Results and Discussion

Germline and Somatic Sequence Variants in CLSPN

We resequenced all coding exons of *CLSPN* from EBV-immortalized lymphoblastoid cell lines established from 71 index cases with a family history of cancer. Germline variants of *CLSPN* were present among lymphoblastoid cell lines in 1 of 25 (4%) probands from breast cancer families, and in 1 of 46 (2%) probands from multicancer families (Table 1). We therefore extended our analysis to search for *CLSPN* mutations in a panel of sporadic cancer cell lines representing diverse tumor types, including breast cancer. Nonsynonymous sequence variants were uncovered in 5 of 52 (10%) sporadic cancer cell lines representing eight distinct tumor types (Fig. 1). These were confined to cell lines derived from tumors of the breast (2 of 17 lines), ovary (1 of 10 lines), and hematopoietic system (2 of 5 lines; Table 1). All variants were missense mutations, none of which was present among a series of at least 140 control individuals (280 alleles), nor were they reported to be single nucleotide polymorphisms (Table 1). Among the six *CLSPN* variants found within sporadic cancer cell lines, five occurred in the heterozygous state. An exception was the breast cancer cell line, MDA-MB-175, which was homozygous for a variant encoding the P956L substitution. A second cell line, ES2, derived from an ovarian tumor, contained two independent missense mutations leading to the R1184W and I783S substitutions. Sequencing of cloned reverse transcription-PCR products generated from ES2 revealed that the substitutions were biallelic, occurring *in trans*. The R1184W variant was also present within a leukemia cell line, MOLT4, but was absent among 184 healthy individuals, suggesting that this may represent a recurrent tumor-associated mutation. The CLSPN I236V, I783S, A1146S, and R1184W variants each affected amino acid residues that are conserved between human and *Xenopus*, suggesting that they may be important for proper functional activity.

Table 1. *CLSPN* Mutations among Cancer Families and Sporadic Cancer Cell Lines

Sample	Phenotype	Nucleotide change	Amino acid change	Frequency in population controls
Germline of cancer families (n = 71)				
Case 1	Breast cancer	A>G, nt 706	I236V	0/546
Case 3	LFL	G>T, nt 3436	A1146S	0/184
Sporadic cancer cell lines (n = 52)				
MDA-MB-415	Breast cancer	G>A, nt 1091	G364D	0/551
MDA-MB-175	Breast cancer	C>T, nt 2867	P956L	0/551
ES-2	Ovarian cancer	C>T, nt 3550	R1184W	0/184
		T>G, nt 2348	I783S	0/140
MOLT4	Leukemia	C>T, nt 3550	R1184W	0/184
RPMI-8226	Multiple myeloma	A>G, nt 1361	H454R	0/165

NOTE: Nucleotide and amino acid positions are based on GenBank accession no. GI:21735568.
Abbreviations: LFL, Li-Fraumeni–like; nt, nucleotide.

Sequence Variant Within ATRIP

Given our observation of CLSPN variants within a subset of sporadic cancer cell lines, we extended our screen to search for somatic mutations within genes encoding proteins that have a direct functional interaction with CLSPN, i.e., *ATR* and *ATRIP*. We previously reported the absence of mutations within *CHK1*, which encodes a CLSPN-interacting protein, within this same panel of cell lines (6). Here, a single nonsynonymous sequence variant was identified within *ATRIP* from the panel of 52 tumor-derived cell lines. This consisted of a heterozygous missense mutation (c.2297C>T), predicted to encode an amino acid substitution ATRIP-T766M within the ovarian cancer cell line OVCAR3. This affects a residue that is immediately proximal to the conserved PIKK interaction motif within ATRIP, although it does not itself display cross-species sequence conservation (33). The *ATRIP* variant was not detected within the germline of 241 healthy control individuals (482 alleles), indicating that it is not a common polymorphism within the general population.

Functional Properties of CLSPN Mutants

The relatively high frequency of sequence variants detected within *CLSPN*, compared with the frequency of *ATRIP* mutations within the same set of cell lines, prompted us to examine the effect of the *CLSPN* variants on function. To evaluate the functional consequences of each CLSPN variant, we examined their ability to undergo phosphorylation in response to DNA damage, as well as their effect on DNA damage–induced phosphorylation of CHK1. We first confirmed that phosphorylation of CHK1 at Ser^{345} is reproducibly observed in U2OS cells (Fig. 2A), as well as in the breast cancer cell lines MDA-MB-436, MDA-MB-453, and MDA-MB-468 (Fig. S1) in

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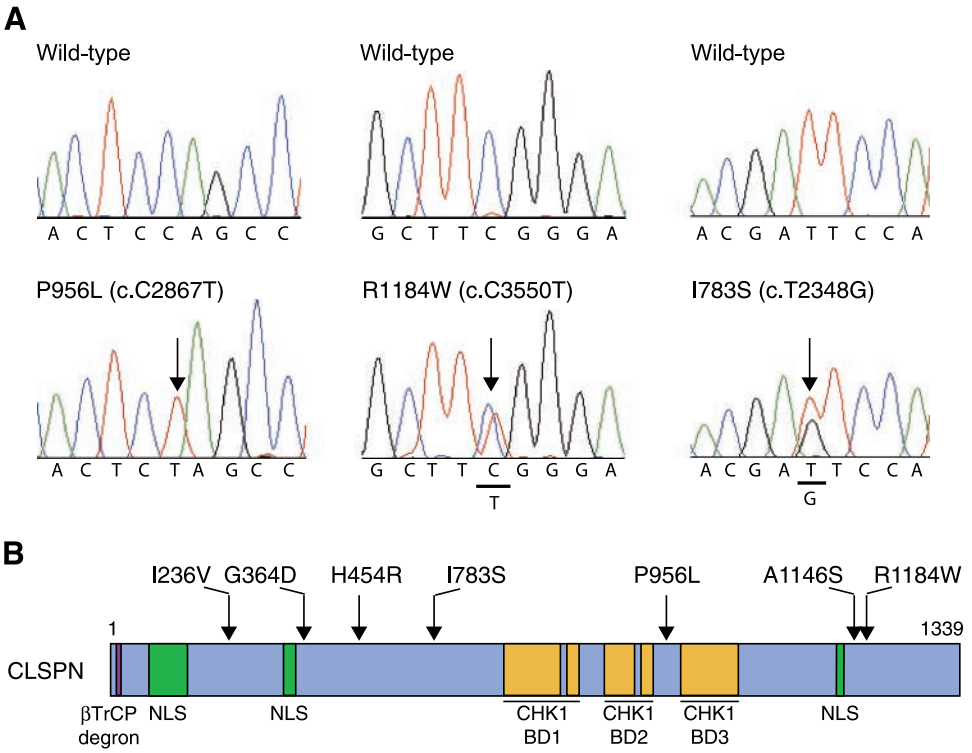

FIGURE 1. Somatic and germline variants of *CLSPN*. **A.** Nucleotide sequence traces depicting missense mutations (*arrows*) within the breast cancer cell line MDA-MB-175 (*P956L*), the leukemia cell line MOLT4 (*R1184W*), and the ovarian cancer cell line ES-2 (*I783S*). Wild-type sequences from control individuals are shown for comparison (*top*). **B.** Schematic representation of the CLSPN protein, indicating the location of variants in relation to known functional domains. βTrCP recognition motif DSGxxS (*βTrCP degron*), potential nuclear localization signals (*NLS*), CHK1-binding domains 1 to 3 (*CHK1-BD1, CHK1-BD2*, and *CHK1-BD3*).

response to either UV or hydroxyurea exposure. SiRNA-mediated knockdown of endogenous *CLSPN* in U2OS cells specifically suppressed this effect (Fig. 2A). The siRNA constructs were designed to target the 3′ untranslated region (UTR) of the native *CLSPN* transcript, making it possible to simultaneously express ectopic *CLSPN* constructs lacking the 3′UTR. Ectopic 3′UTR–truncated CLSPN was subject to physiologic regulation, as shown by its phosphorylation following hydroxyurea exposure (Fig. 2B). We then engineered U2OS cells by knocking down endogenous *CLSPN*, while ectopically expressing variants were identified in our mutation screen. We established two independent reconstitution assays in U2OS cells to investigate the possible functional defects of CLSPN mutants. In the first, we tested CHK1 phosphorylation post-transfection of siControl and siCLSPN oligos following UV treatment. We chose to examine cells following UV treatment because this form of DNA damage gave the most robust induction of CHK1 phosphorylation in siControl transfectants (Fig. 2A). In this reconstitution assay, wild-type CLSPN displayed a robust restoration of CHK1 phosphorylation following UV treatment. Of six variants tested, one (CLSPN-I783S) reproducibly displayed a reduced complementation of endogenous CLSPN knockdown (Fig. 2C), as measured by CHK1 phosphorylation across three independent experiments. CLSPN has previously been implicated in a pathway that prevents premature chromatin condensation in response to hydroxyurea-induced DNA damage (20). Therefore, we established a second reconstitution assay in which we evaluated the survival of U2OS cells posttransfection of siControl and siCLSPN oligos and following hydroxyurea treatment. CLSPN-I783S reproducibly displayed a reduced complementation of endogenous CLSPN knockdown, as determined by cell viability, following treatment with hydroxyurea (Fig. 2D). All other mutant constructs tested (I236V, A1146S, P956L, G364D, and R1184W) showed a level of complementation similar to the wild-type construct and therefore are currently of unknown functional significance (data not shown). Although we have not identified a cell line with definitive homozygous inactivation of CLSPN, the ES2 ovarian cancer cell line harbors two heterozygous mutations in *CLSPN*, occurring *in trans*, i.e., the I783S and R1184W mutations. This cell line showed very low levels of CHK1 phosphorylation following UV or hydroxyurea treatment (Fig. S2). Taken together, these observations suggest that CLSPN-I783S encodes an attenuated allele, potentially linked to tumorigenic properties. However, in the absence of assays that directly measure CLSPN activity, it remains possible that additional mutants identified in this study may encode proteins with attenuated activity beyond the level of detection by our assays. Similar effects have been noted for the CHEK2-I157T variant, which encodes an attenuated protein that is impaired in only certain aspects of the CHEK2-mediated DNA-damage response (34-36). Alternatively, some *CLSPN* mutations uncovered within our screen may be passenger mutations rather than driver mutations in human tumorigenesis. As has been noted in other studies, robust

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functional assays are an absolute requirement to distinguishing between passenger and driver mutations (37).

In addition to the *CLSPN* variants observed in sporadic cancer cell lines, we also detected germline variants of this gene within EBV-immortalized lymphoblastoid cell lines established from probands from two cancer families. However, extended genetic analysis of matched tumor DNA for loss-of-heterozygosity, or of germline DNA from an affected relative for cosegregation, provided no evidence for an association of the variant alleles with disease susceptibility in these particular families (data not shown). This is consistent with recent analyses of breast cancer families which also found no evidence for linkage of *CLSPN* to breast cancer predisposition (38, 39). Candidate gene analysis is complementary to genome-wide association studies, especially for rare variants in genes that are known to play a role in DNA-damage pathways. As illustrated by a number of genes studied to date (7, 8, 10), multiple different variants, each of which is relatively rare in the population, may contribute toward cancer risk, without the ancestral founder effects that are best identified by genome-wide association studies. As such, combining mutational analysis with detailed functional assays is particularly valuable in dissecting pathways that, in aggregate, contribute toward breast cancer susceptibility in the general population.

In conclusion, our findings have highlighted a potential role for mutational disruption of *CLSPN* in human tumorigenesis. Given that CLSPN is a multifunctional protein and that it modulates, but does not directly execute the phosphorylation of ATR substrates, it is perhaps not surprising that some functional defects of the CLSPN mutants have not been revealed by the specific *in vitro* functional assays that we used. As our understanding of the precise biochemical activity of CLSPN improves, additional assays may become available to shed further light on the functional effect of the germline and somatic mutations described here. In the meantime, resequencing of this gene among primary tumors of the breast, ovary, and hematopoietic system may be required to more precisely determine the extent and role of *CLSPN* disruption in these malignancies.

Materials and Methods

High-Risk Cancer Cohorts

Our cohort was comprised of EBV-immortalized lymphoblastoid cell lines established from probands with early-onset breast cancer and a family history of disease ($n = 25$; ref. 40), Li-Fraumeni syndrome ($n = 5$), or variant Li-Fraumeni syndrome ($n = 41$). Breast cancer families were not linked to

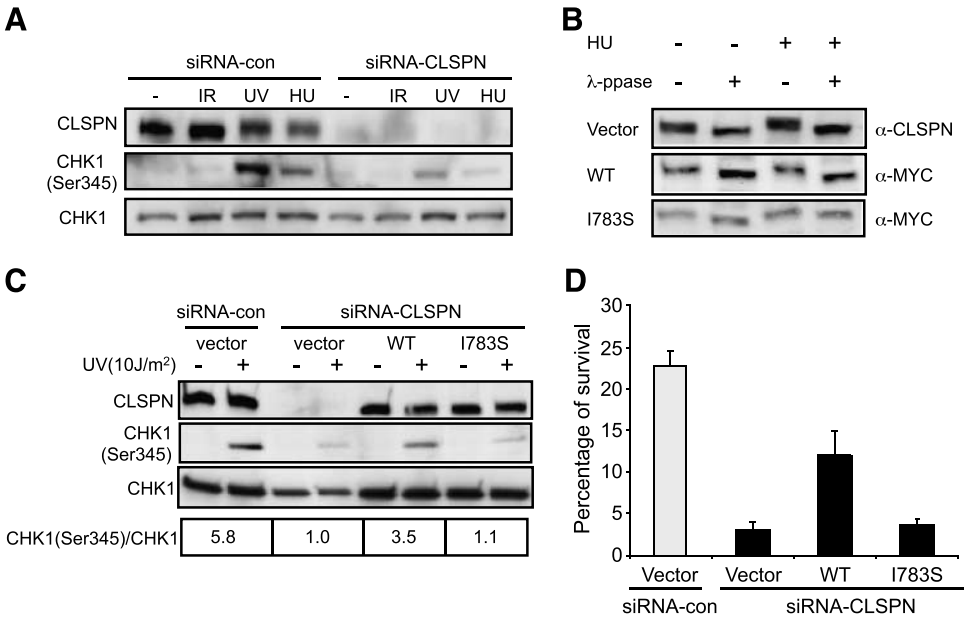

FIGURE 2. The *CLSPN*-I783S variant encodes a functionally defective protein. **A.** siRNA-mediated knockdown of endogenous *CLSPN* in U2OS cells is associated with reduced levels of phosphorylated CHK1. U2OS cells transfected with control (*con*) siRNA, or *CLSPN*-3′UTR–specific siRNA were untreated or treated with IR (10 Gy), UV (10 J/m^2), or hydroxyurea (2 mmol/L). Cells were harvested 1 h posttreatment followed by immunoblotting to measure CLSPN, phosphorylated CHK1 (Ser^{345}), and total CHK1 protein levels. **B.** *In vivo* phosphorylation of CLSPN-I783S, following exogenous expression in U2OS cells, is not impaired. U2OS cells infected by adenoviral constructs expressing myc-tagged wild-type (*WT*) or mutant (*I783S*) CLSPN were either treated with hydroxyurea (2 mmol/L), or left untreated. Western blots of γ-protein phosphatase (γ-ppase)–treated or untreated lysates were probed with an anti-CLSPN antibody to measure total levels of CLSPN expression, or with an anti-MYC antibody to measure ectopic CLSPN levels. **C.** *In vivo* phosphorylation of CHK1 by CLSPN-I783S is impaired. U2OS cells were cotransfected with CLSPN-3′UTR–specific siRNAs to knock down the endogenous protein, as well as with constructs exogenously expressing wild-type or I783S constructs lacking a 3′UTR. Cells were divided into two plates and treated with 10 J/M^2 UV, or left untreated. Cells were harvested 1 h posttreatment and subjected to immunoblotting with antibodies against CLSPN, phosphorylated CHK1 (Ser^{345}), or total CHK1. Protein levels were quantified by phosphorimaging and the amount of phosphorylated CHK1, relative to total CHK1, determined (*bottom*). **D.** Wild-type CLSPN, but not CLSPN-I783S, partially rescues the hydroxyurea sensitivity of CLSPN-depleted U2OS cells. CLSPN-depleted U2OS cells were cotransfected with exogenous DNA constructs expressing wild-type CLSPN or CLSPN-I783S. Transfected cells were plated at a low density and treated with 2 mmol/L of hydroxyurea for 24 h. After 2 wk, the number of colonies was determined by counting. Columns, mean number of cells surviving hydroxyurea treatment relative to untreated cells; experiments were done in triplicate.

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germline mutations in *BRCA1* or *BRCA2*. Likewise, multicancer families were not linked to germline mutations within *p53*. All clinical material was collected under appropriate Institutional Review Board–approved protocols. EBV-immortalized lymphoblastoid cell lines were maintained in Iscoves modified Dulbecco's medium supplemented with 20% fetal bovine serum, L-glutamine (2 mmol/L), and penicillin (10 units)/streptomycin (10 μg) at 37°C in 5% CO_2.

Sporadic Cancer Cell Lines

Sporadic cancer cell lines were obtained from American Type Culture Collection and were comprised of the following: breast (MCF7ADR, MDA-MB-435, T47D, BT483, MDA-MB-436, MDA-MB-453, MDA-MB-468, MDA-MB-415, MDA-MB-231, MDA-MB-175, MDA-MB-157, HS157, HS467T, HS496T, HS578T, UACC893, BT549), ovarian (ES-2, IGROV-1, MDAH2774, OV1063, OVCAR3, OVCAR4, OVCAR5, OVCAR8, SKOV3, SW626), lung (NCIH460, NCI522, HOP92), CNS (SF295, SNB19, U251), hematopoietic [CCRF-CEM (acute lymphoblastic leukemia), K562 (chronic myelogenous leukemia), MOLT4 (acute lymphoblastic leukemia), RPMI-8226 (multiple myeloma), and SR (large-cell lymphoma)], colon (COLO205, HCT116, HCT15), renal (786-0, ACHN, CAKI-1, SN12C, U031), melanoma (LOXM-VII, M14, SKMEL2, UACC62), and osteosarcoma (U20S, SAOS2). Cell lines were not authenticated after receipt. Cell lines were grown in either DMEM or RPMI 1640 (hematopoietic cell lines) supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), and penicillin (10 units)/streptomycin (10 μg) at 37°C in 5% CO_2.

Control Populations

EBV-immortalized lymphoblastoid cell lines were established from 200 anonymous blood donors with no previous diagnosis of cancer, the majority of whom were Caucasian, through the Massachusetts General Hospital blood bank. A second, multiethnic, control series consisted of DNA extracted from 360 healthy individuals with approximately equal representation of African-American, Caucasian, Hawaiian, Japanese, and Latino ethnic subgroups (41).

Mutational Analysis

Genomic DNA was extracted from cell lines using a standard phenol-chloroform procedure followed by ethanol precipitation and resuspension in TE buffer. All coding exons of *CLSPN* and *ATRIP* were amplified from genomic DNA using intronic primers. Primers and PCR conditions are available upon request. PCR amplicons were purified by exonuclease I (U.S. Biochemical) and shrimp alkaline phosphatase (U.S. Biochemical) treatment according to the recommendations of the manufacturer. Purified amplicons were diluted and sequenced using the BigDye terminator kit version 1.1 in conjunction with an ABI3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were analyzed for the presence of mutations by visual inspection, and with the use of Sequence Navigator and Factura software (Applied Biosystems) to mark and display heterozygous or homozygous positions. All nonsynonymous sequence variants were confirmed from at least two independent genomic DNA amplifications. Denaturing high-performance liquid chromatography using the Wavemaker System (Transgenomic) was used to screen for variants within the coding sequence of *ATR*. Wavemaker software (Transgenomic) and the Stanford prediction program[5] were used to determine the optimal annealing temperatures for mutational analysis by denaturing high-performance liquid chromatography. Amplicons exhibiting anomalies by heteroduplex analysis were sequenced to determine the underlying nucleotide change.

Genotyping of Population Controls

Nonsynonymous sequence variants were genotyped within population controls by direct nucleotide sequencing (up to 200 Caucasian controls) as described above, or using a Sequenom MassArray system (up to 360 multiethnic controls). Primers and probes were designed for each variant using the SpectroDesign software and are available on request. Multiplex PCR was done in 5-μL volumes that contained 0.1 units of *Taq* polymerase (Amplitaq Gold, Applied Biosystems), 5 ng of genomic DNA, 2.5 pmol of each primer, and 2.5 mol of deoxynucleotide triphosphate. Thermocycling was at 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 30 s. Unincorporated deoxynucleotide triphosphates were deactivated using 0.3 units of shrimp alkaline phosphatase (Roche), followed by primer extension using 5.4 pmol of each primer extension probe, 50 μmol/L of the appropriate deoxynucleotide triphosphate/dideoxynucleotide triphosphate combination, and 0.5 units of ThermoSequenase (Amersham Pharmacia). Reactions were cycled at 94°C for 2 min, followed by 40 cycles of 94°C for 5 s, 50°C for 5 s, and 72°C for 5 s. After the addition of a cation-exchange resin to remove residual salt from the reactions, ~7 nL of the purified primer-extension reaction was loaded onto a matrix pad (3-hydroxypicolinic acid) of a SpectroCHIP (Sequenom). SpectroCHIPs were analyzed using a Bruker Biflex III MALDI-TOF mass spectrometer (SpectroREADER, Sequenom) and the spectra processed using SpectroTYPER (Sequenom). Genotyping percentage exceeded 98% for each assay. Error rates as previously assessed by duplicate samples on this platform have been estimated at 0.3%.

Generation of CLSPN Expression Constructs

A COOH-terminal myc-tagged construct expressing full-length, wild-type *CLSPN* was generated by PCR and cloned into the pCDNA3.1/V5-His TOPO TA expression vector (Invitrogen). Site-directed mutagenesis was used to generate mutant constructs expressing the naturally occurring Ile236Val, Ala1146Ser, Pro956Leu, Gly364Asp, Ile783Ser, or Arg1184Trp *CLSPN* variants. The integrity of all constructs was verified by nucleotide sequencing. The expression of each construct was confirmed by Western blot analysis using an anti-MYC antibody after transfection into U2OS cells. Constructs encoding the wild-type *CLSPN* and each of the *CLSPN* variants were subcloned into the pAd shuttle vector. Briefly, constructs in the pCDNA3.1/V5-His Topo vector were digested with *Bam*HI and *Xho*I and cloned into the *Bgl*II/*Xho*I site of the pShuttle-CMV vector. Recombinant adenoviral plasmids were generated by homologous recombination in *Escherichia coli* and adenovirus titers measured using the Adeno-X titer kit

[5] http://insertion.stanford.edu/melt.html

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(BD Biosciences Clontech), according to the instructions of the manufacturer.

siRNA and DNA Transfections

Duplexes targeting *CLSPN*-3′UTR, or a control transcript, were obtained from Dharmacon, Inc. The coding strand of the *CLSPN*-siRNA was AUUGCAGACAGAAAUUCCAdTdT and the control-siRNA was UCCAGUGAAUCCUUGAGGUdTdT (20). SiRNA duplexes were transfected into U2OS cells using OligofectAMINE (Invitrogen). Briefly, cells were grown to 30% to 40% confluency and transfected with siRNAs at 100 nmol/L for 48 h, after which time, the culture medium was replaced and cells were allowed to recover at 37°C with 5% CO_2 for 24 h. The siRNA transfection was then repeated. In cotransfection experiments, U2OS cells were initially transfected with siRNA alone using OligofectAMINE (Invitrogen). After 24 h, cells were transfected with *CLSPN*-expressing constructs using LipofectAMINE 2000 (Invitrogen). After a 24-hour recovery period, cells were mock-treated or UV-treated (10 J/m^2).

Immunoblotting

U2OS cells transfected with control or *CLSPN*-3′UTR siRNAs were treated with IR (10 Gy), UV (10 J/m^2), or hydroxyurea (2 mmol/L), or left untreated, 48 h after the second siRNA transfection. Cells were harvested for Western blotting after 1 h by lysis in NETN buffer consisting of 150 mmol/L of NaCl, 1 mmol/L of EDTA, 20 mmol/L of Tris (pH 8), 0.5% Nonidet P40, and 1× protease inhibitor cocktail (Roche). Lysates were either untreated, or treated with λ phosphatase (400 units) for 30 min at 30°C in the buffer provided (New England Biolabs), and resolved on a 7.5% SDS/PAGE gel. For immunoblot analysis, proteins were transferred to polyvinylidene difluoride membranes (Millipore), probed with the appropriate antibody, and visualized with the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer). Antibodies used were the anti-CLSPN (ab3720) antibody (Abcam), the anti-CHK1 (G-4) antibody (Santa Cruz Biotechnology), the antiphosphorylated CHK1 (Ser^{345}) antibody (Cell Signaling Technology), anti-Myc (9B11) antibodies (Cell Signaling Technology), and the β-tubulin antibody (Upstate).

Colony Formation Assay

U2OS cells cotransfected with control or *CLSPN*-3′UTR siRNA were plated at low density and treated with 2 mmol/L of hydroxyurea for 24 h, at which time, cells were washed and the culture medium replaced. Colonies were stained with Coomassie blue and counted after 2 wk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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