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Tumor-Associated Mutations in Caspase-6 Negatively Impact Catalytic Efficiency

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Abstract

Unregulated, particularly suppressed programmed cell death is one of the distinguishing features of many cancer cells. The cysteine protease caspase-6, one of the executioners of apoptotic cell death, plays a crucial role in regulation of apoptosis. Several *CASP6* gene somatic mutations in tumor tissues have been reported. This work explores the effect of *CASP6* tumor-associated mutations on the catalytic efficiency and structure of caspase-6. In general, these mutations showed decreased overall catalytic turnover. Mutations within 8-Å of the substrate-binding pocket of caspase-6 were found to be the most catalytically deactivating. The R259H substitution was found to be the most inactivating among caspase-6 tumor-associated variants. This substitution disrupts the cation- π stacking interaction between Arg-259 and Trp-227, which is indispensable for proper assembly of substrate-binding loops in caspase-6. Sequence conservation analysis at the homologous position across the caspase family suggests a role of this cation- π stacking in the catalytic function of caspases generally. These data suggest that caspase-6 deactivating mutations may contribute to the multifactorial carcinogenic transformation.

TOC image

Keywords

caspase-6; cysteine protease; apoptosis; cancer

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INTRODUCTION

Caspases are a family of cysteine-aspartic proteases that are critical to mediating programmed cell death and maintaining homeostasis within the cell. Caspases are synthesized as inactive procaspase zymogens consisting of a connected prodomain, large subunit, intersubunit linker region, and small subunit (Figure. 1A). Depending on their function within the apoptotic cascade, caspases are classified as initiators (caspase-2, -8, -9) or executioners (caspase-3, -6, -7)^{3, 4}. When the cell receives a death signal, either from cellular stress (intrinsic pathway) or an extracellular death ligand (extrinsic pathway), initiator caspases are recruited to the apoptosome (caspase-9) or DISC complex (caspase-8) to begin the signal cascade.⁵ The constitutively dimeric executioner caspases are activated when cleaved at the intersubunit linker by the initiator caspases. Active executioner caspases proteolytically cleave substrates, which are both structural and repair proteins, at critical aspartic acid residues, which can activate or deactivate the substrates. Both the extrinsic and intrinsic apoptotic pathways eventually lead to the destruction of the cell.⁶⁻⁹

Caspase-6 is expressed as an inactive zymogen that is activated by proteolytic processing at a conserved aspartate residue in the intersubunit linker (Asp-179 (caspase-3 processing) or Asp-193 (self-activation)) between the large and small subunits and in the prodomain.¹⁰ Caspase-6 is often activated by caspase-3 rather than by initiator caspases¹¹ and has been shown to undergo self-activation both *in vitro* and *in vivo*^{12, 13}. Caspase-6 is less apoptotic than either caspase-3 and -7, which are directly processed by initiators caspase-8 and -9¹⁴. However, the overexpression of caspase-6 alone has been shown to promote cell death¹⁵. Structurally, caspase-6 has the closest homology with caspase-3 and caspase-7, but has a preference for cleaving VEID motifs rather than the DEVD consensus sequence favored by caspase-3 and -7¹⁶. Furthermore, caspase-6 has distinct substrates compared to other caspases, such as lamin A/C¹⁷. An extensive analysis of caspase-2 and caspase-6 native substrates using MS-based proteomics revealed 204 unique caspase-6 substrates¹⁸. Many caspase-6-specific substrates are known to be tumor-associated based on their inclusion in the census of human cancer genes¹⁹. Representative cancer-associated caspase-6 substrates include the double-strand-break repair protein, RAD21; the cellular tumor antigen p53, TP53; the breast cancer type 1 susceptibility protein, BRCA1; the DNA repair protein complementing XP-G cells, ERCC5; the DNA mismatch repair protein, MLH1; and the ras-related protein Rab-10, RB10. Thus, the crosstalk of caspase-6 with these known tumor-associated substrates suggests the potential involvement of caspase-6 in development of cancer.

As mediators of apoptotic cell death, caspases natively play an essential role in preventing the development of cancer. Unregulated cell proliferation and reduced apoptotic index are hallmarks of cancer, and caspase inhibition can lead to a reduced apoptotic index, thereby promoting tumorigenesis²⁰. Inhibited expression of proapoptotic molecules and the development of inhibitory somatic mutations could diminish cancer cell apoptosis. Somatic mutations in caspases have been identified in tumor-associated tissues^{2, 21-28}. For example, *CASP8* somatic mutations were found in advanced gastric cancer carcinoma that significantly decreased caspase-8 apoptotic activity²⁹. The well-known MCF-7 breast cancer cell line lacks caspase-3 expression due to a functional deletion mutation in the *CASP3*

gene³⁰. Importantly, these somatic mutations evidence a decrease in caspase activity when compared to wild type and suggest that caspase inhibition may contribute to cancer pathogenesis.

In addition, caspase-6 showed differential gene regulation profiles in various tumor tissues, which have been tested and catalogued in the Catalogue of Somatic Mutations in Cancer (COSMIC database)¹ (Figure 1B). Both overexpression and underexpression of caspase-6 have been observed across various tumor and tumor-associated tissues. For various tumor tissues, an aberrant gene regulation profile usually correlates with the observations of a mutant *CASP6* genotype (Figure 1B). There were exceptions to this pattern, particularly in the tumor-associated samples from the adrenal gland, central nervous system (CNS), hematopoietic/lymphoid, lung, and the soft tissues, in which changes in gene regulation patterns did not correlate with enhanced mutational frequency in the *CASP6* gene. Although several *CASP6* mutations have been detected in tumor samples, only 0.13% of the total 29,665 tumor tissue samples showed mutations in *CASP6*. While the identities of these mutations in *CASP6* in tumor tissues have been reported, the effect of each mutation on caspase-6 function is completely understudied. Together, these mutations and atypical gene expression profiles may implicate caspase-6 in tumorigenesis.

Perhaps due to its diminished apoptotic role, caspase-6 has not been as highly scrutinized for its role in cancer pathogenesis as other caspases. Initial work to compile a list of *CASP6* somatic mutations in tumor-associated tissues was reported more than a decade ago². Three *CASP6* somatic mutations were detected in gastric and colorectal cancer tissues and were classified to be missense and nonsense mutations, but no studies reporting functional characterization of the proteins resulting from mutant *CASP6* have been reported. A search of the COSMIC database¹ revealed that caspase-6 harbored several mutations in tumor tissues. Of all reported mutations in *CASP6*, 64% resulted from missense mutations that encoded for a different amino acid after a single nucleotide change in the codon of *CASP6* gene (Figure 1C). Caspase-6 also harbored functionally deleterious mutations including deletions (frameshifts) and nonsense mutations resulting to a premature appearance of a stop codon within the *CASP6* gene. Interestingly, a significant fraction (27%) also showed synonymous substitution (silent) mutations. These tumor-associated mutations, with emphasis on nonsense and missense mutations, are distributed across the structure of caspase-6, impacting regions both within the active site cavity and substrate-binding loops (Figure 1D) and outside, suggesting potential of these mutations to modulate caspase-6 function.

To further explore the impact of somatic mutations on the function of caspase-6, caspase-6 tumor-associated variants were produced and tested in the present study. Selected *CASP6* somatic mutations identified from the COSMIC database¹ and from the initial *CASP6* mutational analysis study² were selected. *CASP6* mutations from the COSMIC database were chosen based on their confirmed somatic status, their mutation type, and their varied structural location within the enzyme (Figure 1D). Based on these criteria, seven caspase-6 tumor-associated variants were generated with amino acid changes located in the prodomain, large, and small subunit. This panel allowed us to explore the trend of somatic *CASP6* mutations in cancer pathogenesis and associated decreased apoptotic activity.

MATERIALS AND METHODS

Database Analysis of CASP6 Gene Mutations

CASP6 gene mutations in tumor samples were identified from COSMIC database (<http://cancer.sanger.ac.uk/cosmic>)¹ and from a report of *CASP6* gene mutations in colorectal and gastric carcinomas.² Assessment of a total of 29,815 sample tissues across all tumor-associated tissue types was reported for *CASP6* gene mutations. Representative missense mutations were selected based on the location of these mutations in the caspase-6 structure which spans within the region of the prodomain (M19R), the 90's region (L98F), within the dimer interface (M208T and K285N), at the base of the helix adjoining L4 (E247K), and within 8Å radius of the active site Cys-163 (R259H and P276S). At the time we initiated this work, the M208T, E247K and K285N mutations were present in the COSMIC database. These mutations were not present in the release of the COSMIC database available on the date of publication. Based on conversations with a curator at the COSMIC database, we came to understand that some of the data in the COSMIC database derives from the International Cancer Genome Consortium (ICGC). The ICGC freshly recompiles all of the data before each release of COSMIC. This means that all of the individual contributing labs are required to submit all of their data for each subsequent release. If the lab no longer exists, forgets to submit data or if an analysis algorithm changes, legitimate data contained in a prior release may not appear in a current release. Thus there are number of reasons that the caspase-6 mutations we have studied, M208T (COSM201127), E247K (COSM585487), K285N (COSM345581) may not be included in the current ICGA release. In short, the absence of these mutations from the current release does not necessarily invalidate them.

Generation of Caspase-6 Tumor-Associated Variants

The caspase-6 variants, full length wild type (FL WT), FL C163S and constitutively two-chain (CT) were derived from the synthetic, *E. coli* codon-optimized (His)₆ C-terminally tagged caspase-6 gene (Celtek Bioscience) that were ligated into the NdeI/BamHI sites of pET11a vector.³¹ Selected caspase-6 tumor-associated variants were generated using FL WT as template through Phusion® site-directed mutagenesis (Thermo Scientific™). The caspase-6 R259H CT and W227A CT variants were particularly generated using caspase-6 D179CT as the template for the site-directed mutagenesis.

Caspase-6 Protein Expression and Purification

Caspase-6 expression constructs were transformed into the BL21(DE3) T7 express strain of *E. coli* (New England Biolabs). Overnight seed cultures were initially grown in 2xYT medium supplemented with 0.1 mg/ml ampicillin (Sigma) at 37 °C. Dense cultures were then diluted 1,000-fold with 2xYT containing 0.1 mg/ml ampicillin and shaken at 37 °C until A₆₀₀ reached 0.6. Protein expression was induced by the addition of 1mM isopropyl 1-thio-β-D-galactopyranoside and cultures were shaken at 20 °C for 18 h. Cells were centrifuged at 4,700 × *g* for 10 min at 4 °C and stored at -20 °C until use. Freeze-thawed cells were lysed using a microfluidizer (Microfluidics, Inc.) in lysis buffer (50 mM Tris, pH 8.5, 300 mM NaCl, 5% glycerol, 50 mM imidazole) and centrifuged at 30,600 × *g* for 1 h at 4 °C. The supernatant was loaded into a 5-ml HiTrap nickel affinity column (GE Healthcare) and washed with lysis buffer until the absorbance returned to baseline levels. These proteins

were eluted with elution buffer (50 mM Tris, pH 8.5, 300 mM NaCl, 5% glycerol, 250 mM imidazole) and diluted 5-fold with buffer A (20 mM Tris, pH 8.5, 2 mM DTT) to reduce the salt concentration. These protein samples were then loaded into a 5-ml HiTrap Q HP column (GE Healthcare). The chromatographic separations were performed with linear NaCl gradient, and each protein was eluted in 20mM Tris, pH 8.5, 200mM NaCl, 2mM DTT. These eluted proteins were stored at -80°C until use. Purified caspases were analyzed by SDS-PAGE to confirm its identity and purity.

Caspase-6 Activity Assays

To measure caspase-6 activity, the activity of 100 nM purified caspase-6 (WT and tumor-associated variants) was assayed over 7 min at 37°C in caspase-6 activity assay buffer (100 mM HEPES, 120 mM NaCl, 0.1% CHAPS, 10% sucrose, 5 mM DTT). For substrate titration, a range of 0-500 μM fluorogenic substrate VEID-AMC [*N*-acetyl-Val-Glu-Ile-Asp-(7-amino-4-methyl-coumarin), Enzo Life Sciences Inc.] was used. Fluorescence kinetic measurements ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 365nm/495nm) were performed as two independent trials on two separate days in 100- μL reactions in a 96-well format using a microplate reader (SpectraMax M5, Molecular Devices). Initial velocities versus substrate concentration were fit to a rectangular hyperbola using GraphPad Prism (GraphPad Software, San Diego, USA) to determine the kinetic parameters K_{M} and k_{cat} . Enzyme concentrations were determined by active-site titration with the quantitative covalent inhibitor VEID-CHO (*N*-Acetyl-Val-Glu-Ile-Asp-aldehyde; Enzo Life Sciences Inc.). Caspase-6 was added to inhibitor solvated in DMSO in 96-wells V-bottom plates at room temperature for 1.5 h in caspase-6 activity assay buffer. Aliquots (90 μL) were transferred in duplicate to black-well plates and assayed with 50-fold molar excess of substrate. The protein concentration was determined to be the lowest concentration at which full inhibition was observed and was thus used to calculate k_{cat} .

Self-Proteolysis Assay of Caspase-6 Tumor-Associated Variants

Caspase-6 variants (3 μM) were allowed to self-cleave in caspase-6 activity assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 120 mM NaCl, and 5 mM DTT) at 37°C for 24 h. SDS loading buffer was added to the samples and boiled for 10 mins prior to analysis by 16% SDS-PAGE. The gels were imaged using a ChemiDocTM MP imaging system (Bio-Rad).

Proteolysis of Procasapse-6 FL C163S Protein by Caspase-6 Tumor-Associated Variants

Catalytically inactive procaspase-6 FL C163S (3 μM) was incubated with each of caspase-6 tumor-associated variants (0.3 μM) in caspase-6 assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 120 mM NaCl, and 5 mM DTT) at 37°C for a predetermined time interval. SDS loading buffer was added to the samples and boiled for 10 mins prior to analysis by 16% SDS-PAGE. The gels were imaged using ChemiDocTM MP imaging system (Bio-Rad).

Intrinsic Fluorescence Spectroscopy

Caspase-6 tumor-associated variants (3 μM) in 20 mM phosphate buffer, pH 7.5, 120 mM NaCl, and 2 mM DTT were prepared. Fluorescence emission scans (305–400 nm) were

collected after excitation at 280 nm or 295 nm using a J-1500 spectrometer (Jasco) equipped with fluorescence emission monochromator (FMO-522) and detector (FDT-538). The signal was acquired by setting the emission detector at high-tension voltage value of 700 V, the data integration time (D.I.T) to 1 s and data pitch to 1 nm. The bandwidths used for excitation and emission was 2 nm and 10 nm, respectively.

Caspase-6 Full-length Structural Model Generation

The full-length caspase-6 zymogen model (residues 1–293) shown in Figure 1D was built from crystal structures of the caspase-6 zymogen. In our model, chain A was derived from PBD code 4IYR (chain A); chain B of this model was derived from PDB code 3NR2 (chain A) as templates. The missing residues ([1–30, 174–186, and 292–293] in PBD code 4IYR (chain A); [1–30, 167–186, 261–271, and 292–293] in PBD code 3NR2 (chain A)) were built by *de novo* modeling using Chimera/Modeller platforms.^{32, 33} All illustrations with molecular visualization were generated using the PyMOL Molecular Graphics System (Schrödinger, LLC).

RESULTS AND DISCUSSION

Caspase-6 mutants identified in tumor tissues have decreased activity

To further investigate the effect of these mutations on caspase-6 function, seven caspase-6 tumor-associated mutations were selected based on their distribution across the caspase-6 structure (highlighted in *bold* in Figure 1D). All selected *CASP6* mutations had a confirmed somatic (non-germline) status and we focused our interrogation on less conservative mutations. Representative missense mutations were selected from within the region of the prodomain (M19R), the 90's helix (L98F), within the dimer interface (M208T and K285N), at the base of the helix adjoining L4 (E247K), and within 8-Å radius of the active site Cys-163 (R259H and P276S). At the time of publication, the M208T, E247K, and K285N mutations had been removed from the COSMIC database for unknown reasons (see Materials and Methods). The caspase-6 variants were expressed in *E. coli* and purified. The activities of the caspase-6 tumor-associated variants were assessed using a fluorogenic peptide-based substrate, VEID-AMC (Table 1). These variants were observed to have 2–457 fold decreases in catalytic efficiency compared to the wild type (WT) caspase-6. Mutations within the substrate-binding groove, P276S and R259H, showed the most negative impact on caspase-6 activity. The decrease in the catalytic efficiency of all the caspase-6 tumor-associated variants against peptide-based substrates suggests that the mutations have caused changes in the structure or dynamics that result in less effective substrate catalysis.

Caspase-6 is the sole caspase for which a mechanism for intramolecular self-cleavage has been observed.^{12, 13} As is the case under certain human conditions, during bacterial expression, WT caspase-6 is capable of self-maturation.³⁴ All of the caspase-6 tumor-associated variants were overexpressed in *E. coli* for 18 h at 20 °C and purified in the same manner (described in the Materials and Methods). These expression conditions led to various extents of self-activation of WT and mutant caspase-6, as evidenced by the appearance of the large and small subunits following cleavage at D193, which is the activating event. Typically, caspase-6 is proteolytically cleaved at three sites: one site in the

prodomain (Asp-23) and two sites (Asp-179, Asp-193) flanking the linker region (Figure 1A) to reach full maturation. In the context of cancer, the crucial activating event is cleavage at Asp-193. Complete removal of the intersubunit linker following cleavage at Asp-179 has no impact of measured catalytic function and removal of the prodomain also has no impact on catalytic function.³¹

All caspase-6 cancer mutants were able to undergo self-activation, though L98F and K285N were able to cleave their own intersubunit linker at Asp-179 even faster than WT (Figure 2A). While this increase in activity is observable for self-processing it appears that it may not be more rapid in the context of caspase-6 activity towards peptide substrates (Table 1) or protein substrates (Figure 2B).

The caspase-6 tumor-associated variants expressed from the gene for full-length caspase-6 were allowed to undergo self-proteolysis *in vitro* by 24-hr incubation in caspase-6 activity assay buffer at 37 °C (Figure 2B). As expected, the full-length wild type (FL WT) caspase-6 showed a higher fraction of the fully cleaved large and small subunits, indicating close to full maturation. The caspase-6 phosphomimetic variant, S257D, is a surrogate of a phosphorylated caspase-6 at position 257. The S257D substitution is inactivated due to a misaligned substrate-binding groove in the crystal structure of this variant of caspase-6³⁴ and correspondingly, showed no further cleavage into its large and small subunits (Figure 2B). L98F showed similar cleavage pattern as FL WT at 24 h suggesting that L98F mutation did not dramatically impact the self-cleavage activity over this time course. R259H and P276S showed higher fractions of large subunits with attached intersubunit linker, a cleavage pattern different from the FL WT and L98F, which indicates that these variants have somewhat slowed self-cleavage activity. Thus, the accessibility of the intersubunit linker to caspase-6 appears to be different in both P276S and R259H, perhaps suggesting an altered geometry of the substrate-binding cleft, generating a less proteolytically active enzyme.

The *trans* (intermolecular cleavage) activities of selected caspase-6 tumor-associated variants were tested using procaspase-6 FL C163S, a catalytically inactive variant of caspase-6, as a protein substrate (Figure 2C). Consistently, both P276S and R259H showed less proteolytic activity against this protein substrate compared to L98F and WT caspase-6 (Figure 2C). R259H emerged as the least active variant in cleaving procaspase-6 FL C163S, underscoring the severity of its impact on the proper assembly of the substrate-binding cavity for efficient proteolysis. As the most inactive among caspase-6 tumor-associated variants in cleaving both peptide-based and protein substrates, we sought to assess whether the R259H impacted function in a homogenous preparation of the fully mature form of caspase-6. To achieve this, the R259H mutation was then introduced into a constitutively two-chain (CT) caspase-6 expression construct³¹ that allows independent expression of the large and small subunits, mimicking a fully cleaved and mature caspase-6. The two-chain R259H CT is dramatically inactivated for cleavage of protein substrates compared to the mature, two-chain (CT) wild-type caspase-6. This result further suggests that the R259H mutation negatively impacts the activity of the mature (cleaved) caspase-6 in cleaving protein substrates (Figure 2D). Together, these data suggest that the majority of inactivating mutations in caspase-6 found in tumor-associated tissues may have acquired a misaligned substrate binding cleft or other defect-introducing structural changes that decrease in caspase-6 catalytic efficiency. Thus, it

appears that the impairment of caspase-6 function in tumors need only prevent caspase-6 activity against other substrates, whereas self-cleavage poses no significant threat to the viability (apoptosis avoidance) of the cancer cells. This is consistent with our emerging understanding of caspase-6 activation. We hypothesize that procaspase-6 self-activation is critical in a neurodegenerative context, but activation by upstream caspases is critical for apoptosis.

Caspase-6 R259H disrupts interaction necessary for proper assembly of substrate-binding groove

R259H is the most inactivating mutation among caspase-6 tumor-associated variants we analyzed. To further understand the impact of the R259H substitution on proper assembly of the substrate-binding groove, the crystal structure of active and mature caspase-6 (PDB code 2WDP) was further inspected. Arg-259, which is located at the base of L4, is part of a network of H-bond and cation- π stacking interactions (Figure 3A) that provides a stable foundation for proper assembly of substrate binding loops and is therefore essential for the establishment of a catalytically competent caspase-6. The usually positively-charged guanidinium group of arginine exhibits a high propensity to stack against aromatic residues and is also capable of H-bonding to neighboring oxygen atoms.³⁵ In caspase-6, Arg-259 forms two H-bonds with Asp-231 and interacts with Trp-227 through cation- π stacking, as predicted by the CAPTURE analysis which can identify energetically significant cation- π interactions.³⁶ Further, Trp-227 also holds L3 in place through a cation- π stacking interaction with His-219, underscoring its importance in situating the active site loop bundle. Mutating Trp-227 to alanine produced a predominantly inactive, full-length caspase-6 (Figure S1A) that was incapable of self-cleavage even after incubation for 24 h at 37 °C (Figure S1B). To assess its ability to cleave other substrates, W227A was expressed in the mature, two-chain version of caspase-6 using the CT construct. Caspase-6 W227A CT was unable to cleave the fluorogenic peptide substrate VEID-AMC (Table 1), demonstrating that this substitution is deleterious to both self-activation in *cis* and cleavage of other substrates in *trans*. This strongly suggests that the Arg-259 interaction with Trp-227 through cation- π stacking is critical for function. Both residues interacting with Arg-259 (His-219 and Trp-227) are part of the S4 substrate-binding pocket in caspase-6. This cation- π interaction (Arg-259 and Trp-227 in caspase-6) is conserved in six of the seven apoptotic caspases but not the inflammatory caspases (Figure 3B). Thus, this interaction may also be used by other apoptotic caspases to stabilize the substrate-binding groove for efficient substrate binding and catalysis. On the other hand, the Asp-231 position is not conserved across the caspase family, so the two H-bonds between Arg-259 and Asp-231 are unique to caspase-6. These residues also form a salt bridge, which would additionally be disrupted by the R259H mutation. Thus, the Arg-259 mutation is likely to impact caspase-6 through disruption of two critical interactions with Trp-227 and Asp-231. One might expect, based on this analysis that tumor-associated mutations might occur at Trp-227 and Asp-231. To date, tumor-associated mutations at any of these three positions (Trp-227, Asp-231, Arg-259; caspase-6 numbering) have not been observed in other caspases, however this may be simply due to incomplete coverage of all tumor genotypes.

The intrinsic fluorescence properties of Trp-227 allow this residue to function as a sensitive probe for the cation- π stacking interaction between Arg-259 and Trp-227 and the other network of interactions that give rise to the proper assembly of the substrate-binding groove. Caspase-6 has only two Trp residues, and they are both located in the substrate binding L1 (Trp-57) and at the base of L4 (Trp-227), which is directly adjacent to Arg-259. Trp-57 in both the zymogen and active forms are constantly solvent-exposed while the microenvironment of the Trp-227 varies between these forms of caspase-6 (Figure 3A). Thus, intrinsic tryptophan fluorescence will likely report the relative microenvironment of Trp-227 between the absence and presence of R259H mutation and in turn, provides structural hints as to the proper assembly of the substrate-binding groove of caspase-6. Intrinsic tryptophan fluorescence is dependent on the polarity of the environment, where shifts in the maximum emission towards longer wavelengths (red shift) indicate higher solvent exposure. Intrinsic fluorescence spectra were collected from caspase-6 variants including the catalytically inactive procaspase-6 FL C163S, the mature caspase-6 (CT), and the mature caspase-6 R259H (CT).

As expected, upon specific tryptophan excitation at 295 nm (Figure 3C), the procaspase-6 zymogen showed a 10-nm shift in maximum emission towards a shorter wavelength (*blue* shift) compared to the two-chain mature caspase-6 wild type (CT) suggesting a less solvent-exposed Trp-227 microenvironment. In the procaspase-6 zymogen structure (Figure 3A), the intersubunit linker sits on top of the substrate-binding groove of procaspase-6 zymogen restricting the solvent accessibility of Trp-227, one of the critical residues in the substrate-binding pocket of caspase-6. Meanwhile, the R259H CT variant displayed a significantly extended 12-nm shift in maximum emission towards longer wavelength (*red* shift) compared to caspase-6 CT indicating a highly solvent-exposed Trp-227 microenvironment. A similar trend was observed with 280 nm excitation of all aromatic residues (Trp, Tyr, Phe) (Figure 3D). Thus, the change in the polarity of the Trp-227 microenvironment in the presence of the R259H mutation in caspase-6 suggests a disruption of the stabilizing cation- π stacking interaction of Arg-259 with Trp-227. It further disrupts the networks of H-bonding interactions that results in misaligned loops in the substrate binding pocket, rendering it incapable of efficient catalytic turnover of substrates.

CONCLUSIONS

This study demonstrates that all of the selected mutations in caspase-6 found in several tumors resulted in an overall decrease in the catalytic efficiency of caspase-6. The fact that all of the selected mutations in all regions of caspase-6 had functional impacts strongly suggests that diminishing caspase-6 intrinsic activity via somatic mutations is helpful in establishing a tumorigenic phenotype. In particular, R259H was found to be severely deactivating via a mechanism that disrupts the cation- π stacking and the networks of H-bonding interactions necessary for proper alignment of loops in the substrate-binding groove of caspase-6.

One would anticipate that mutations in caspases would decrease the apoptotic potential of the cells containing mutant caspases. Neutralizing/inactivating mutations in caspase-6 should therefore play a role in tumorigenesis. For example, caspase-6 is a direct activator of

the initiator caspase-8.³⁷ Thus, in cellular context, activation through caspase-6 presents one of the regulatory pathways of caspase-8. *CASP8* is a tumor-associated gene and its polymorphisms are correlated with increased risk for various types of cancer.^{38–41} In addition to its central role in apoptosis, caspase-8 has emerging nonapoptotic roles in cells such as potentiating NF- κ B signaling,⁴² regulating autophagy,⁴³ and cellular adhesion and migration.^{44, 45} Thus, regulation of caspase-6 may possibly impact both the apoptotic and the nonapoptotic tumor-associated roles of caspase-8, suggesting another possible mechanism for caspase-6 to play a role in tumor development. While these data suggest that caspase-6 mutations contribute to development of a cancerous phenotype, we do not anticipate that mutations in caspase-6 alone would be sufficient to cause cancer independently. In fact, a recent analysis of genetic variations of over 60,000 exomes found a rare R259H-encoding allele of caspase-6.⁴⁶ It is likely that caspase-6 mutations act in concert with many other mutant alleles, ultimately contributing to a cancerous condition. Therefore, R259H alone could be present in many healthy cells, but may predispose individuals to certain types of cancer.

Together with the aberrant regulation of caspase-6, some tumor samples that harbored *CASP6* gene mutations also contained mutations in other caspases (Table 2). Particularly interesting is the co-existence of mutations in the initiator *CASP8*, a known oncogene,⁴⁷ where 24% of the tumor samples with *CASP6* mutations also harbored *CASP8* mutations. The co-occurrences of mutations in multiple caspases may be expected and underscore the complexity of each individual event of tumorigenesis. This may suggest the existence of altered regulation of the apoptotic cascade or other nonapoptotic pathways relevant to the evolution of a tumor phenotype.

The fact that several different mutations in different regions of caspase-6 were observed in different tumors suggests the possibility that unique contributions of various aspects of caspase-6 function may be at play in different cancers. The roles of each component in the pathway to tumor development will enable a better understanding of the controlling factors that allow the misregulation observed in cancer cells of many varieties. This work demonstrates that in cases when caspase-6 is mutated in tumors, significant impacts on catalytic function are observed. What remains to be seen is whether other aspects of caspase-6 regulation, such as post-translational modifications, dynamics and substrate selection are likewise impacted by these tumor-associated mutations. One might envision that delivery of WT caspases, as we have recently demonstrated,⁴⁸ might allow recovery of the deficiencies in caspase-6 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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visible only for one of the monomers in this orientation. The model procaspase-6 structure was generated based on zymogen structures 4IYR and 3NR2 with missing regions modeled *de novo*. Substrate-binding loops (L1, L2, L3, L4) along with key regions in caspase-6 (90's, 130's and the active site) are also indicated. Highlighted ***bold*** are the tumor-associated mutations interrogated in this study. An asterisk (*) indicates a premature appearance of stop codon characteristic of a nonsense mutation.

Figure 2.

Most caspase-6 tumor associated variants showed decreased proteolytic activity for self-processing and protein substrates. (A) Cleavage pattern of caspase-6 tumor-associated variants expressed in *E. coli* and purified to homogeneity. All caspase-6 tumor-associated mutations were introduced into the full-length (FL) caspase-6 wild-type (WT) construct in a pET11a bacterial expression vector. Active procaspases self-process at Asp-193 to generate the large subunit with the intersubunit linker attached (Lg+L) and small subunit (Sm). Subsequent cleavage at Asp-79 results in removal of the intersubunit linker to generate the

large subunit (Lg). Concomitant cleavage at Asp-23 results in removal of the N-terminal prodomain (N) residues 1-23. (B) Self-cleavage pattern of recombinant caspase-6 tumor-associated variants (3 μ M) before and after 24-h incubation in caspase-6 activity assay buffer at 37 °C (left) and the associated quantification (right). (C) Proteolysis of catalytically inactive procaspase-6 C163S (3 μ M) by caspase-6 tumor-associated variants (0.3 μ M) before and after 24-h incubation in casapse-6 activity assay buffer at 37 °C (left) and the associated quantification (right). (D) Proteolysis of catalytically inactive procaspase-6 C163S (3 μ M) by caspase-6 tumor-associated variant, R259H CT (0.3 μ M) after incubation in caspase-6 activity assay buffer at 37 °C for the indicated times (left) and the quantification (right). Two-chain forms of the mature wild type (CT) and R259H CT caspase-6 variants were generated to allow independent expression of the large and small subunits without the need for proteolytic cleavage for its activation.

Figure 3.

R259H disrupts interactions necessary for proper assembly of the substrate-binding groove. (A) The crystal structure of the mature, fully cleaved and unliganded caspase-6 (PDB code 2WDP) (*left*). The location of Arg-259, the active site Cys-163, and the two tryptophan residues (Trp-57 and Trp-227) in caspase-6 are indicated. *Inset view* highlights the cation- π stacking and networks of H-bond interactions in which Arg-259 are involved, as well as the substrate binding loops (L3, L4) and substrate-binding pocket (S4) (*middle*). The crystal structure of procaspase-6 zymogen (PDB code 3NR2) and the location of Arg-259, Trp-51, Trp-227, and the intersubunit linker (*cyan*) are indicated (*left*). (B) The amino acid sequence alignment of key residues within the network of interactions of Arg-259 across caspase family including both apoptotic and inflammatory caspases. (C) Intrinsic tryptophan fluorescence profiles of full length procaspase-6 C163S, caspase-6 CT, and caspase-6 CT. Excitation at 295 nm primarily probes the polarity of the tryptophan microenvironment. (D) Intrinsic fluorescence profiles of caspase-6 variants excited at 280-nm wavelength. Excitation at 280 nm probes the polarity of the microenvironment of all aromatic residues in a protein including tryptophan, tyrosine and phenylalanine.

Table 1

Kinetic parameters of selected caspase-6 tumor variants identified from *COSMIC* database¹ and other sources².

Caspase-6 mutation	K_M (μM)	k_{cat} (s^{-1})	$10^3 \times k_{cat}/K_M$ ($\text{M}^{-1}\text{s}^{-1}$)	Fold decrease in turnover <i>versus</i> WT
WT	34 ± 3.6	1.1 ± 0.03	32	1
M19R	163 ± 19	0.80 ± 0.04	5.0	6.40
L98F	46 ± 4.2	0.68 ± 0.02	15	2.10
M208T	170 ± 12.0	1.5 ± 0.04	8.8	3.60
E247K	84 ± 10	1.6 ± 0.06	19	1.70
P276S	314 ± 26	0.57 ± 0.02	1.8	18.0
R259H	577 ± 135	0.04 ± 0.01	0.07	457
K285N	50 ± 4.0	0.18 ± 0.01	3.6	8.90
W227A	ND	ND	ND	ND

Kinetic parameters of caspase-6 tumor-associated variants were determined using fluorogenic substrate, VEID-AMC using 100-nM enzyme in caspase-6 activity assay buffer. *Error*, represents standard error of the mean (S.E.M.) of two independent experiments. ND = not detectable

Table 2

Identified missense and nonsense mutations in caspase-6 from various tumor tissue types and its corresponding tissue distribution. Data were pooled from the COSMIC database (Forbes et al., 2011) and from a reported *CASP6* mutational study (Lee et al., 2006). Highlighted in **bold** are the specific caspase-6 tumor-associated variants interrogated in this study.

Mutation	Tissue	Histology	Other Caspase Mutations ⁺	Reference
H11Y	Prostate	Carcinoma	none	(Robinson et al., 2015)
G14D	Skin	Carcinoma	none	(Pickering et al., 2014)
M19R	Ovary	Carcinoma	none	(CGARN ^{**} , 2011)
D23V	Bone	Ewings sarcoma	none	(Crompton et al., 2014)
R28K	Skin	Carcinoma	caspase-8 (S33F, S375F)	(Sharpe et al., 2015)
M30I	Endometrium	Carcinoma	caspase-2 (A259A)	(Forbes et al., 2011)
A34T ^{**}	Endometrium, Stomach	Carcinoma	caspase-3 (L118I, D192N); caspase-4 (R55W); caspase-10 (E22D); caspase-14 (E49K)	(Forbes et al., 2011)
R44K	Colon	Carcinoma	caspase-3 (F193L); caspase-8 (R162I)	(Lee et al., 2006)
C68F	Kidney	Carcinoma	none	(Durinck et al., 2015)
L98F	Endometrium	Carcinoma	caspase-1 (frameshift)	(Forbes et al., 2011)
L98H	Thyroid	Carcinoma	none	(Forbes et al., 2011)
K99E ^{**}	Liver	Carcinoma	none	(Forbes et al., 2011)
K99T	Cervix	Carcinoma	caspase-8 (N197T); caspase-14 (Q70K)	(Forbes et al., 2011)
H101R ^{**}	Liver	Carcinoma	none	(Forbes et al., 2011)
V106I	Cervix	Carcinoma	none	(Forbes et al., 2011)
V115A	Stomach	Carcinoma	caspase-5 (frameshift)	(Wang et al., 2014)
D146E	Liver	Carcinoma	none	(Forbes et al., 2011)
P174S	Skin	Carcinoma	none	(Sharpe et al., 2015)
E183D [#]	Endometrium	Carcinoma	caspase-7 (K109N); caspase-8 (R52I, R111I, T461P, T503P)	(Forbes et al., 2011)
E191K	Urinary tract	Carcinoma	none	(Forbes et al., 2011)
M208T	Large Intestine	Carcinoma	none	(Forbes et al., 2011)
R220W	Endometrium	Carcinoma	caspase-2 (L232R, R417W, L125F); caspase-3 (R207 [*]); casp-4 (V252I); caspase-5 (Y87 [*] , Y116 [*]);	(Forbes et al., 2011)
E247K	Lung	Carcinoma	none	(Forbes et al., 2011)
E247 [*]	Breast	Carcinoma	caspase-4 (E351A); caspase-8 (D73A, D132A, E399 [*] , E441 [*])	(Forbes et al., 2011)
R259C	Endometrium	Carcinoma	caspase-1 (G85W); casp-2 (A412V); caspase-4 (K42Q); caspase-7 (T18A); caspase-8 (E36 [*] , E95 [*])	(Forbes et al., 2011)
R259H	Colon	Carcinoma	none	(Lee et al., 2006)
R260 [*]	Large Intestine	Carcinoma	none	(Forbes et al., 2011)
P276S	Breast	Carcinoma	none	(Forbes et al., 2011)
A279V [#]	Endometrium	Carcinoma	caspase-7 (K109N); caspase-8 (R52I, R111I, T461P, T503P)	(Forbes et al., 2011)
K285N	Lung	Carcinoma	none	(Forbes et al., 2011)

* Nonsense mutations identified in caspase-6.

^{**} Mutation found in two separate samples.

^{*/#} Mutations found in the same sample ID.

⁺ Other caspase mutations found in the same sample ID.

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