

A candidate tumor suppressor HtrA1 is downregulated in ovarian cancer

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We report here that HtrA1, a candidate tumor suppressor, is downregulated in ovarian cancer. Expression of HtrA1 is downregulated in five of seven ovarian cancer cell lines. In total, 59% of primary ovarian tumors have either a complete absence or markedly reduced levels of HtrA1 expression compared to the brushings of ovarian surface epithelium. Primary ovarian tumors show high frequencies of loss of an allele at microsatellite markers near *htrA1* locus on 10q26. Downregulation of HtrA1 in SKOV3 by antisense transfection promotes anchorage-independent growth, while exogenous expression of HtrA1 in OV202 induces cell death. HtrA1-induced cell death is not inhibited by the broad caspase inhibitor, zVAD(O-Me)fmk, but instead reflects serine protease activity associated with HtrA1. These observations raise the possibility of HtrA1 as a candidate tumor suppressor involved in promoting serine-protease-mediated cell death and that downregulation of HtrA1 in ovarian cancer may contribute to malignant phenotype.

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Introduction

Ovarian cancer is one of the leading causes of gynecological-related deaths among women in the US. Of the 27 000 women diagnosed each year with this disorder, over half die of their disease (Greenlee *et al.*, 2000). These statistics highlight the need for improved understanding of the pathogenesis of this neoplasm.

Like cancers of other tissues, ovarian cancer is considered to result from an accumulation of a series of genetic alterations. Alterations in tumor-suppressor genes such as *p53* (Kohler *et al.*, 1993), *pRB* (Li *et al.*, 1991), and *NOEY2* (Yu *et al.*, 1999), and oncogenes such as *K-ras* (Enomoto *et al.*, 1991), *c-myc* (Katsaros *et al.*,

1995), and *HER-2/neu* (Ross *et al.*, 1999) have been shown to play an important role in ovarian carcinogenesis (Orsulic *et al.*, 2002). To search for additional alterations that might play a role in the biology of ovarian cancer, we recently generated suppression subtraction hybridization (SSH) cDNA libraries between normal ovarian epithelium and primary tumors. One of the differentially expressed genes identified from this screen (Shridhar *et al.*, 2002) was *htrA1*, a human homologue of bacterial *htrA/DegP* gene product.

HtrA1 belongs to the HtrA family of serine proteases that is well conserved from bacteria to humans. The bacterial HtrA gene product is one of the most well-characterized proteins of the HtrA family and its presence is necessary for bacterial thermotolerance (Clausen *et al.*, 2002). It has recently been shown that bacterial HtrA has dual roles, acting as a chaperone at normal temperature and as an active protease at high temperature (Spiess *et al.*, 1999; Krojer *et al.*, 2002).

Human HtrA1 was originally isolated from fibroblasts as a transformation-sensitive protein due to its downregulation by SV40 (Zumbrunn and Trueb, 1996). It contains an N-terminal insulin-like growth factor binding protein (IGFBP) domain, a Kazal-type trypsin inhibitor motif, and C-terminal trypsin-like protease and PDZ domains (Hu *et al.*, 1998). It is downregulated in malignant melanoma, and stable overexpression of HtrA1 inhibited proliferation in metastatic melanoma cell line (Baldi *et al.*, 2002).

To validate and extend our previous analysis indicating that HtrA1 is downregulated in all four ovarian tumor SSH libraries (Shridhar *et al.*, 2002), we examined the expression of HtrA1 in ovarian cancer cell lines and primary tumors by RT-PCR, Northern blot, Western blot, and Light-Cycler analyses. Furthermore, we investigated the functional significance of HtrA1 downregulation in an ovarian cancer cell line with endogenous HtrA1 expression, and the effect of HtrA1 re-expression in an ovarian cell line with low levels of HtrA1.

Results

HtrA1 is expressed in normal ovary and downregulated in ovarian cancer

Hybridization with the full-length coding sequence of HtrA1 to a multiple tissue Northern blot revealed that

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HtrA1 is widely expressed. Particularly noteworthy in the present context is the expression in ovary (Figure 1a). Further analysis revealed that HtrA1 is expressed in ovarian surface epithelial (OSE) cells (Figure 1b), the normal counterpart of ovarian cancer. Notably, compared to immortalized normal OSE cells, expression of HtrA1 is lost or markedly diminished in several ovarian cancer cell lines when analysed by RT-PCR and Western blot (Figure 1b and 1c, respectively). Northern blot analysis of primary ovarian tumors (Figure 1d) indicates that HtrA1 is lost or reduced in both early- and late-stage tumors compared to normal ovarian epithelial cell brushings. Semiquantitative RT-PCR (Figure 1e) and Light-Cycler analyses (Figure 1f) confirmed the reduced levels of HtrA1 in several ovarian tumors of different histologies.

HtrA1 expression is aberrantly regulated in several types of cancer

RT-PCR analysis of HtrA1 expression in several types of cancer cell lines and tumors indicates that HtrA1 expression is aberrantly regulated. HtrA1 expression is downregulated in two of 13 primary brain tumors, three (BT474, MDA-MB-361, and UACC893) of eight breast cancer cell lines, two (HepG2 and Hep3B) of 10 liver cancer cell lines, and one (C33-A) of nine cervical cancer cell lines (Figure 2). Identities of other cell lines are listed in the figure legend.

Loss of heterozygosity (LOH) at 10q26 is associated with HtrA1 downregulation

To investigate the mechanism of HtrA1 downregulation, LOH analysis was performed to determine if *htrA1* locus was a target for deletion. Utilizing six genetic markers around the *htrA1* locus, we analysed 39 ovarian tumors and matched normal DNA for LOH. The cytogenetic band locations, base position relative to *htrA1* locus, and the frequency of LOH are shown in Figure 3a. Two of the closest markers, the GT repeat marker (32 kb 5' of exon 1) and the CT repeat marker (102 kb 3' of exon 9), showed 32 and 42% LOH, respectively (Figure 3b). This is the first report of such high frequencies of LOH in this region in ovarian cancer. Representative autoradiographs indicative of LOH at each marker are shown in Figure 3c. No homozygous deletion was detected in the tumor samples tested. To determine if LOH corresponds with loss or reduced levels of expression, HtrA1 expression was analysed in 19 of 39 tumors for which RNA was available. Of nine tumor samples that displayed LOH at CT repeat marker, seven tumors showed loss or reduced HtrA1 expression (Figure 3d). Tumors used in LOH analysis and the LOH score are listed in Table 1. To determine if additional mechanism, such as epigenetic silencing, could also be responsible for loss of HtrA1 expression, the ovarian cancer cell line OV207 was treated with the methyltransferase inhibitor 5-aza-2' deoxycytidine. This treatment led to a dose-

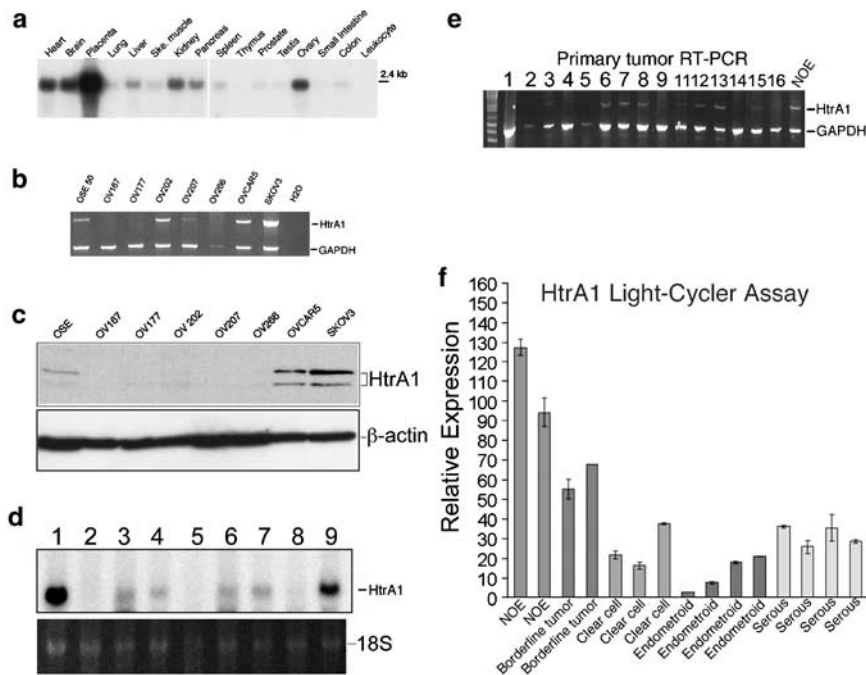


Figure 1 HtrA1 expression is downregulated in ovarian cancer. (a) Northern blot analysis of HtrA1 expression in normal tissues. The highest level of HtrA1 transcripts was detected in placenta followed by brain, heart, and ovary. (b) RT-PCR analysis of ovarian cancer cell lines indicates that HtrA1 is downregulated in four of seven cancer cell lines compared to short-term culture OSE50. (c) Western blot analysis of ovarian cancer cell lines also indicates a similar pattern of downregulation of HtrA1. (d) Diminished HtrA1 expression is observed in seven out of eight primary tumors (1–4, early stages; 5–9, late stages) by Northern blot. (e, f) Semiquantitative and quantitative RT-PCR of primary tumors. Absence or diminished expression of the HtrA1 transcript, compared to normal ovarian epithelial cells (NOE), is detected in primary tumors by semiquantitative RT-PCR (e) and by quantitative Light-Cycler (f). GAPDH is used as a loading control

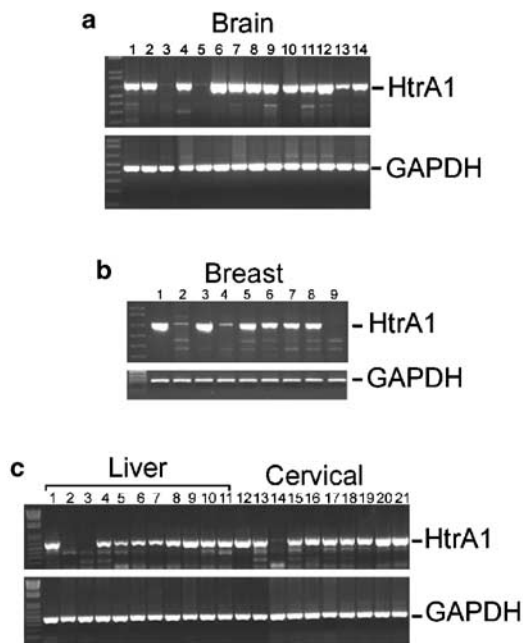


Figure 2 HtrA1 expression is aberrantly regulated in several types of cancer. (a) RT-PCR analysis of HtrA1 in primary brain tumors indicates downregulation of HtrA1 in two of 13 tumors. Lane 1: normal whole brain. (b) HtrA1 expression in downregulated in BT474 (lane 2), MDA-MB-361 (lane 4), and UACC893 (lane 9) breast cancer cell lines. 1, human mammary epithelial cells (HMEC control); 2, BT474; 3, MDA-MB-157; 4, MDA-MB-361; 5, MDA-MB-435; 6, MDA-MB-468; 7, T47D; 8, UACC812; and 9, UACC893. (c) HtrA1 expression is reduced in HepG2 (lane 2) and Hep3B (lane 3) liver cancer cell lines and C-33A (lane 14) cervical cancer cell line. 1, normal liver; 2, HepG2; 3, Hep3B; 4, Huh7; 5, PLC5; 6, SKHep1; 7, SNU182; 8, SNU387; 9, SNU475; 10, SNU423; 11, SNU449; 12, human keratinocyte; 13, C4a; 14, C-33A; 15, Caski; 16, HeLa; 17, HT3; 18, ME180; 19, Ms751; 20, SiHa; and 21, SW756

dependent increase in transcription of HtrA1 (Figure 3e), implicating methylation as another mechanism by which HtrA1 could be downregulated. However, additional experiments are needed to characterize specific sites of methylation regulating HtrA1 expression in ovarian cancer.

Mutational analysis by denaturing high-performance liquid chromatography (DHPLC)

To determine if mutations might also contribute to HtrA1 downregulation, a total of 96 tumor DNAs were analysed for mutations by the DHPLC (Schwartz *et al.*, 1999) using intronic primers (Table 2) flanking the nine exons present in *htrA1*. While single-nucleotide polymorphisms were detected in the intronic regions, no tumor-specific mutations were detected in any of the samples.

Downregulation of HtrA1 promotes anchorage-independent growth

A previous report indicated loss of HtrA1 expression in invasive melanoma and a decrease in matrix invasion *in vitro* following the re-expression of HtrA1 in melanoma

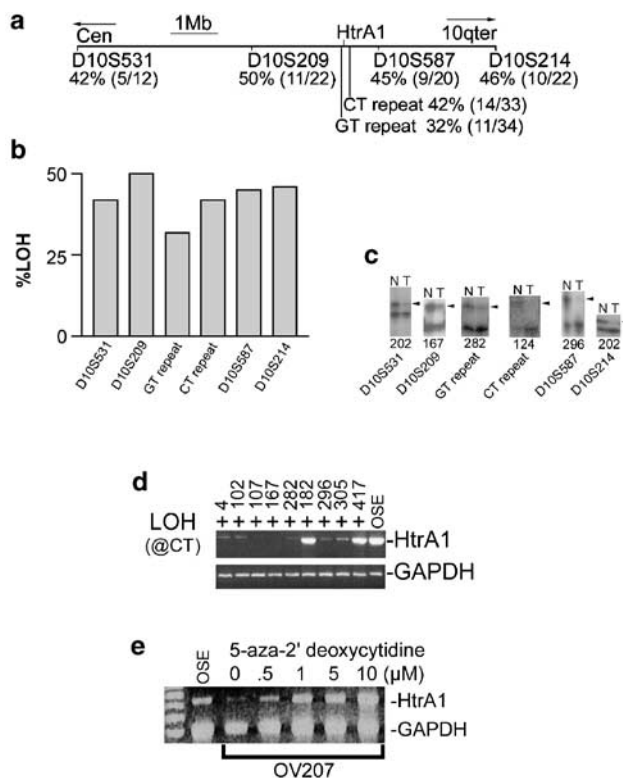


Figure 3 Mechanism of inactivation. (a) Markers used in the LOH studies, corresponding cytogenetic band location, and base position relative to *htrA1* locus. (b) The frequencies of LOH calculated from informative samples at specific markers. (c) The representative LOH autoradiographs for each marker with LOH indicated by arrowheads. (d) LOH at CT repeat corresponds with reduced expression of HtrA1. (e) OV207 was treated with various concentrations of 5-aza-2'-deoxycytidine for 3 days, and HtrA1 expression was analysed by RT-PCR. A dose-dependent increase in HtrA1 expression was observed following this treatment

LM cell line (Baldi *et al.*, 2002), suggesting that loss of HtrA1 may contribute to invasive phenotype. Since invasive phenotype depends not only on matrix migration but also on anchorage-independent growth, the latter was analysed in SKOV3 to determine the functional significance of HtrA1 downregulation in ovarian cancer. Since SKOV3 expresses endogenous HtrA1 (Figure 1c), HtrA1 expression was downregulated by antisense transfection. Of 12 stable clones expressing HtrA1 antisense, four clones displayed efficient downregulation of HtrA1 (Figure 4a, indicated by asterisks); thus they were used in the analysis of soft-agar growth. As shown in Figure 4b and c, downregulation of HtrA1 resulted in a significant increase in the number of colonies grown on soft-agar compared to the vector-transfected controls. These results suggest that downregulation of HtrA1 may contribute to invasive phenotype.

Overexpression of HtrA1 induces cell death

Since HtrA1 also shares a high degree of sequence similarity with the proapoptotic HtrA2 (Suzuki *et al.*, 2001; Hegde *et al.*, 2002; Martins *et al.*, 2002; Verhagen *et al.*, 2002), particularly in the protease domain

Table 1 Loss of heterozygosity (LOH) at 10q26 in ovarian tumors

%LOH	GT-repeat 41.94% (13/31)	CT-repeat 45.7% (16/35)		D10S587 45.0% (9/20)	D10S531 41.7% (5/12)	D10S209 50.0% (11/22)	D10S214 45.5% (10/22)
PCR temp Samples	57°C(-Q)	58°C(+Q)	Samples	57°C(-Q)	55°C(-Q)	55°C(+Q)	55°C(+Q)
4	○	●	4	○	ND	●	○
13	○	○	13	○	UI	○	●
51	●	UI	29	●	○	●	●
90	○	UI	64	ND	ND	●	ND
97	○	○	90	ND	UI	ND	ND
98	○	○	93	●	○	○	○
102	○	●	97	○	UI	●	○
106	○	○	98	●	○	○	○
107	○	●	107	○	UI	●	●
124	●	●	124	UI	UI	●	●
167	UI	●	150	●	UI	●	○
182	UI	●	176	○	○	UUI	○
202	●	ND	182	●	●	●	●
208	○	○	183	○	UI	○	○
209	○	○	202	○	○	○	●
220	●	○	208	ND	UI	●	●
234	○	●	209	○	●	○	○
282	●	●	282	●	UI	●	●
296	●	●	285	●	UI	○	○
305	●	●	354	ND	●	ND	ND
417	UI	●	414	ND	○	ND	ND
461	●	○	417	○	○	●	ND
531	○	UI	453	ND	ND	○	UI
988	○	●	461	○	UI	○	○
990	○	○	496	ND	UI	ND	ND
992	○	○	531	○	●	ND	○
999	●	○	623	ND	UI	ND	○
1002	○	○	647	ND	UI	ND	ND
1004	●	●	684	UI	●	ND	ND
1008	ND	○	276	ND	ND	○	ND
1018	○	○	102	ND	ND	○	ND
1024	UI	●	167	UI	ND	ND	●
1038	UI	○	220	●	ND	ND	UI
1039	UI	○	296	UI	ND	ND	UI
1058	●	●	305	●	ND	ND	●
1097	●	○					
1103	UI	○					
1107	○	●					
1123	●	○					

●: LOH; UI: uninformative.
○: no LOH; ND: not determined.

essential for apoptotic activity, the proapoptotic property of HtrA1 was tested in ovarian cancer cell line OV202. OV202 was selected in this analysis because it expresses very low levels of HtrA1 (Figure 1c). Exogenous expression of HtrA1 in OV202 induced cell rounding and death (Figure 5a, left panel) in a manner similar to that described in HEK293 cells following the transfection of HtrA2 (Suzuki *et al.*, 2001). Moreover, the cell rounding observed with HtrA1 expression in OV202 was not prevented by 100 μM zVAD(OMe)-fmk (fmk[^]N-(N^z-benzyloxycarbonylvalinylalanyl) aspartic acid (O-methyl ester) fluoromethylketone) (Figure 5b), suggesting that this phenomenon may be caspase-independent. In contrast, OV202 cells transfected with the serine protease mutant S328A displayed a normal phenotype (Figure 5a, right panel). A significantly higher percentage of cell death was observed in cells transfected with the wild-type HtrA1 (Figure 5c and d).

This increase in cell death was not observed with the serine protease mutant S328A (Figure 5c and d), suggesting that higher level of cell death was the result of the protease activity.

Discussion

In summary, this report describes the loss of HtrA1 expression in ovarian cancer and demonstrates the effects of HtrA1 downregulation and re-expression in ovarian cancer cell lines. In particular, we present evidence that expression of HtrA1 is reduced in several ovarian cancer cell lines and primary tumors. Moreover, the *htrA1* locus is subject to LOH and epigenetic inactivation. Previous analyses of glioblastoma, prostate cancer, malignant melanoma, and endometrial cancer with multiple markers on 10q have indicated evidence of

Table 2 Primers used in the DHPLC analysis

Primer sequence	Annealing temp (°C)	Product size
Exon 1F 5'-TCACCGCTGCGAGGCCAATG-3'		
Exon 1R 5'-ATGGAGAGAGTGGGGAGCTG-3'	68	656
Exon 2F 5'-CCATGTCTTTCTCTAGGTGG-3'		
Exon 2R 5'-GGTGAAAGCTTCGGAGGTTA-3'	56	212
Exon 3F 5'-AAGGAGCGATGGCTAGGTGT-3'		
Exon 3R 5'-AGAAGTACCTCTGCAGGCG-3'	58	348
Exon 4F 5'-GTCCTGCTTGGTTTTCCAT-3'		
Exon 4R 5'-ACTACAACAAAGTCAGGGGC-3'	56	378
Exon 5F 5'-TTGAACCATGTTATGACACG-3'		
Exon 5R 5'-CCTGCCTCAAAAACAAACA-3'	52	214
Exon 6F 5'-ACCTGCCGGTAACGATTAC-3'		
Exon 6R 5'-CTTTCCCTTCCCCACGTCC-3'	56	252
Exon 7F 5'-TGTACCCTTCTGTGGCCCTT-3'		
Exon 7R 5'-GACACGCATCTGTTACCC-3'	56	195
Exon 8F 5'-GGTGAGAGCTGAGTTTTGCG-3'		
Exon 8R 5'-ACCCACAGGAACACAAG-3'	56	225
Exon 9F 5'-TCTAAGCTGTGCTCTGTCC-3'		
Exon 9R 5'-CAGAGTCTCATCCGTCATC-3'	56	324

two distinct loci for tumor-suppressor genes distal to 10q23 (Albarosa *et al.*, 1996). Although *PTEN* gene on 10q23.3 is probably the more proximal of these two loci, the distal locus is not yet well established. *DMBT1* (deleted in malignant brain tumors 1), mapping to 10q26.13, has been regarded as a potential tumor-suppressor gene representing the distal locus due to the presence of intragenic homozygous deletions and rare mutations (Mollenhauer *et al.*, 1997; Mueller *et al.*, 2002). However, the tumor suppressive role of the gene is still controversial. Two recent reports suggest that *DMBT1* may not be a major target of inactivation in malignant melanoma and glioma (Deichmann *et al.*, 2002; Sasaki *et al.*, 2002). These reports underscore the importance of identifying another candidate tumor-suppressor gene in this frequently deleted region. Human *htrA1* gene maps to chromosome 10q26.13 in close proximity to *DMBT1*. The microsatellite markers used in the LOH analysis are within 10q25.3–10q26.2, encompassing both *htrA1* and *DMBT1*. These markers showed high frequency of deletion in ovarian tumors. Consistent with the notion of HtrA1 as a tumor suppressor, downregulation of HtrA1 in SKOV3 by antisense transfection promoted anchorage-independent growth. Additional support for HtrA1 as a putative tumor suppressor came from the study in malignant melanoma by Baldi *et al.* (2002). HtrA1 expression was significantly lower in autologous lymph node metastases (LM) compared to primary melanomas (Baldi *et al.*, 2002). Stable overexpression of HtrA1 in melanoma LM cell lines attenuated matrix invasion (Baldi *et al.*, 2002), and inhibited proliferation as well as cell growth *in vivo* in nu/nu mice. In addition, the fact that HtrA1 was originally identified as a SV40 transformation sensitive marker lends further support to the potential role of HtrA1 as a tumor suppressor. These observations raised the possibility that HtrA1 could potentially represent the long sought-after distal tumor suppressor in 10q26.

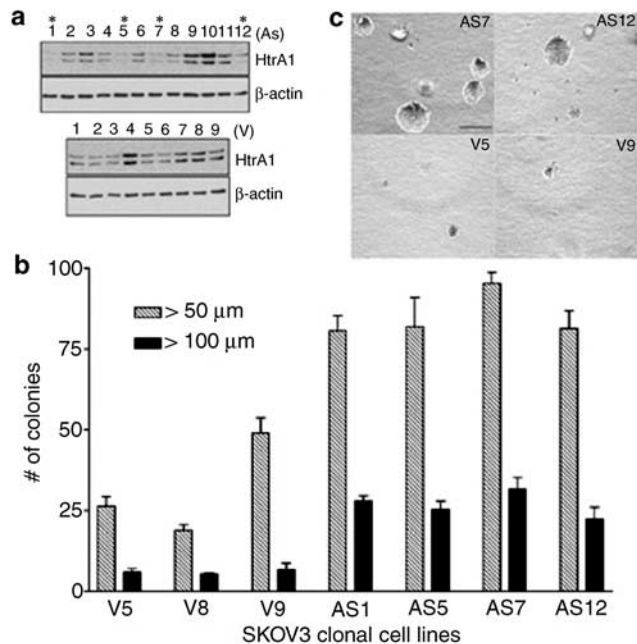


Figure 4 Downregulation of HtrA1 expression promotes the soft-agar growth of SKOV3. (a) HtrA1 expression is downregulated in four of 12 stable clones (upper panel). Clones used in soft-agar growth are indicated by asterisks. HtrA1 expression in nine stable clones transfected with vector control is shown for comparison (lower panel). β -Actin indicates loading control. (b) Downregulation of HtrA1 promotes anchorage-independent growth. A significantly higher number of colonies larger than 50 or 100 μ m are observed in clones expressing antisense HtrA1. (c) A representative photomicrograph of soft-agar growth seen in antisense and vector-control clones

Loss of function of tumor suppressors requires inactivation of both alleles either by homozygous deletions, heterozygous deletions in combination with tumor-specific mutations, and/or epigenetic inactivation by hypermethylation. Our results indicated high frequency of LOH near the gene. However, neither homozygous deletions nor tumor-specific mutations within the coding exons were detected. Instead, our preliminary analysis indicated methylation as a second hit inactivating the function of HtrA1, as evidenced by the induction of transcription after 5-aza-2'-deoxycytidine treatment in the OV207 cell line. Future studies to delineate the specific CpG sites involved in this inactivation are ongoing.

In addition to its role in regulating cell growth, re-expression of HtrA1 induces cell death in ovarian cancer cells. HtrA1-induced cell death is not attenuated by the broad-spectrum caspase inhibitor zVAD(OMe)fmk (Garcia-Calvo *et al.*, 1998), but instead reflects serine protease activity. This is perhaps not unexpected given the fact that other proteases also induce cell death (Williams and Henkart, 1994; Suzuki *et al.*, 2001; Verhagen *et al.*, 2002). In particular, the human homologue HtrA2, which shares extensive homology with the C-terminal protease and postsynaptic density protein 95-Discs large-Zona occludens 1 (PDZ) domains of HtrA1, also induces cell death independent of caspase

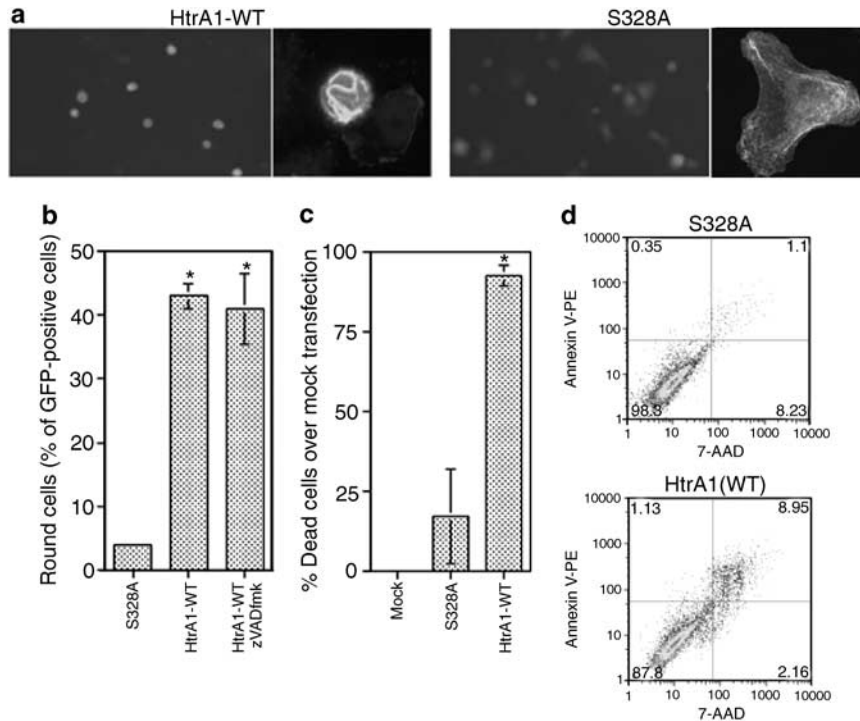


Figure 5 Exogenous expression of HtrA1 induces cell death. (a) OV202 cells were electroporated with 20 μ g of plasmid encoding serine protease mutant HtrA1 (S328A) or wild-type HtrA1 (HtrA1-WT). After 24 h, GFP-positive cells were visualized by fluorescence microscopy. Cells transfected with HtrA1 displayed round morphology, whereas cells transfected with mutant remained flattened. (b) GFP-positive OV202 cells with round morphology were counted, and expressed as the percentage of total GFP-positive cells. (c) Comparison of % cell death (trypan blue positive) induced by S328A and wild-type HtrA1 normalized with cell death from mock transfection. The error bars represent the standard error of means (s.e.m.) calculated from triplicate samples. Asterisks indicate a statistically significant difference from the control as determined by one-way ANOVA ($P < 0.05$). (d) When cell death was analysed with Annexin V-phycoerythrin (PE), a significantly higher percentage of cell death was observed in cells transfected with wild-type HtrA1 (WT) compared to protease mutant HtrA1 (S328A)

activation (Suzuki *et al.*, 2001). In addition to induction of cell death via serine protease, HtrA2, when released from mitochondria during initial steps of apoptosis, also activates caspases by antagonizing inhibitors of apoptosis (IAPs) via its N-terminal tetrapeptide IAP-binding motif (Suzuki *et al.*, 2001; Hegde *et al.*, 2002; Martins *et al.*, 2002; Verhagen *et al.*, 2002). In contrast, HtrA1 does not contain the N-terminal tetrapeptide IAP-binding motif, and therefore it is not expected to be involved in caspase activation. Nonetheless, both HtrA1 and HtrA2 can induce cell death via protease domain independent of caspases, suggesting a potential role of these proteases in caspase-independent cell death. Interestingly, HtrA1 was originally described as a secreted protease, yet it is detected in whole-cell lysates and induces cell death. Further analysis of this apparent paradox is required.

In addition to HtrA2, the identification and cloning of HtrA3 is recently described (Nie *et al.*, 2003). HtrA3 shares high degree of sequence and domain homologies with HtrA1, and therefore it may share functional similarity with HtrA1. Although both HtrA1 and HtrA3 are highly expressed in normal ovary (Nie *et al.*, 2003), semiquantitative RT-PCR analysis of ovarian cancer cell lines indicates that HtrA3 is lost in cell lines that express HtrA1 (data not shown). The differential

expression between HtrA1 and HtrA3 in ovarian cancer cells is intriguing, and necessitates further studies.

The N-terminus of HtrA1 also shares domain homology with the candidate tumor-suppressor Mac25. Mac25 (IGFBP-rP1) is differentially expressed in meningioma, prostate, and mammary cancers and is thought to regulate cell growth by affecting cell-cycle mechanisms (Swisshelm *et al.*, 1995; Baldi *et al.*, 2002). Addition of recombinant Mac25 to the culture medium suppressed the growth of cell lines of diverse origins including cervical and osteosarcomas (Kato, 2000). These data suggest the possibility that the N-terminal domain of HtrA1 may also regulate cell growth in similar fashion. It should be noted that the growth suppression by Mac25 domain might be affecting cell proliferation rather than cell death, since proteolytically inactive S328A mutant containing functional Mac25 domain has no effect in inducing cell death.

Here, we have shown that HtrA1 is consistently downregulated in ovarian cancer. Loss or lower levels of expression in ovarian cancer is due to deletion of an allele due to LOH, thus identifying a new region of deletion for ovarian cancer. Our further studies indicate that HtrA1 may modulate cell death and anchorage-independent growth of ovarian cancer cells. Collectively these results highlight the potential role of HtrA1 as a

tumor suppressor. Further studies that identify molecular targets that interact with HtrA1 will enhance our understanding the role of HtrA1 in normal and malignant cells.

Materials and methods

Cell culture

OV167, OV177, OV202, OV207, and OV266 were low-passage ovarian cancer cell lines established at the Mayo Clinic (Conover *et al.*, 1998), while OVCAR-3, OVCAR-5, and SKOV-3 were purchased from American Type Culture Collection (Manassas, VA, USA). All other cells were grown according to the manufacturer's recommendations.

Tissue processing and tumors

All the tumors were snap-frozen tissues. Tumor contents of the tissues were assessed by hematoxylin and eosin (H&E)-stained sections and verified by a pathologist (Dr Gary Keeney) at the Mayo Clinic. Only tumors with 75–90% tumor content were used for RT-PCR analysis. For control, 20–30 normal ovarian epithelial cell brushings were pooled from patients without cancer and the epithelial nature of these brushings were verified by H&E staining.

Plasmids and antibodies

The plasmids encoding wild-type or mutant S328A HtrA1 were generated by PCR cloning into pcDNA3.1 vector as described previously (Hu *et al.*, 1998). To generate carboxyl terminus GFP fusion construct of HtrA1, PCR products flanking the entire ORF or C-terminal domain corresponding to codons 153–480 of HtrA1 were cloned into pcDNA3.1/CT-GFP TOPO vector. Proper construction of all the plasmids was confirmed by DNA sequencing. Antiserum specific for HtrA1 was raised as described previously (Hu *et al.*, 1998).

Northern blot analysis

In total, 15 µg of total RNA was resolved on 1.2% formaldehyde agarose gels and blotted in 1 × SPC buffer onto Hybond N membranes (Amersham, Piscataway, NJ, USA). The probes were labeled using the random primer labeling system (Invitrogen, Carlsbad, CA, USA) and purified using spin columns (TE100) from Clontech (Palo Alto, CA, USA). Filters were hybridized at 68°C with radioactive probes in a microhybridization incubator (Robbins Scientific, Sunnyvale, CA, USA) for 1–3 h in Express Hybridization solution (Clontech, Palo Alto, CA, USA) and washed according to the manufacturer's guidelines. The probe corresponds to the open reading frame of HtrA1. It does not crosshybridize with HtrA2 or HtrA3.

Immunoblotting

Cell lysates were resolved by SDS-PAGE under reducing conditions, followed by transfer onto 0.45 µm nitrocellulose membrane. After the transfer, immunoblotting was carried out as previously described using affinity-purified polyclonal HtrA antibody (Hu *et al.*, 1998).

Semiquantitative RT-PCR

A total of 50–100 ng of reverse-transcribed cDNA was used in a multiplex reaction with the following primers: HtrA1

forward (5'-TAT CGC GGA CGT GGT GGA GAA GAT CG-3') and HtrA1 reverse (5'-GTC CAG CTC ATG CCT CTG CCT-3') to yield a 595 bp product and GAPDH forward (5'-ACC ACA GTC CAT GCC ATC AC-3') and GAPDH reverse (5'-TCC ACC ACC CTG TTG CTT GTA-3') to yield a 450 bp product. The PCR reactions contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 400 µM of each HtrA1 primer and 50 µM of each GAPDH primer, and 0.5 U of *Taq polymerase* (Qiagen, Valencia, CA, USA) in a 12.5 µl reaction volume. The conditions for amplification were: 94°C for 3 min, then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s in a Perkin Elmer-Cetus 9600 Gene-Amp PCR system. The products of the reaction were resolved on a 1.6% agarose gel. One amplicon was cut from the gel, purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and sequenced to verify the specificity of PCR reactions.

Light-cycler PCR analysis

Using HtrA1F1 (5'-TCC GCA ACT CAG ACA TGG AC-3') and HtrA1R1 (5'-GGC CTC CCG AGT TTC CAT AG-3') plus RPS9F (5'-TCG CAA AAC TTA TGT GAC CC-3') and RPS9R (5'-TCC AGC ACC CCC AAT C-3') primers, duplex PCR amplification was carried out with Light-Cycler (Roche, Indianapolis, IN, USA) in the presence of SYBR-Green dye according to the following conditions: 1 min at 95°C for initial denaturation, followed by 40 cycles at 95°C (10 s), 58°C (15 s), and 72°C (20 s), followed by the measurement of fluorescence at the end of each cycle. After the 40th cycle, melting curve analyses were performed with Light-Cycler software by denaturing the sample at 95°C, rapidly cooling down to 65°C for 15 s, and measuring the fluorescence as the sample temperature was gradually raised to 95°C in the steps of 0.1°C/s. Each run included a negative control.

LOH analysis of primary ovarian tumors

The markers used in this study are listed in Figure 2b, along with their chromosomal locations. The PCR reaction mix contained: 50 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM of each primer, 0.05 µl of [α -³²P]CTP (10 µCi/µl) and 0.5 U of *Taq polymerase* in a 10 µl reaction volume. The conditions for amplification were: 94°C for 2 min, then 30 cycles of 94°C for 30 s, 55–58°C for 30 s, and 72°C for 30 s in a Perkin Elmer-Cetus 9600 Gene-Amp PCR system in a 96-well plate. The PCR products were denatured and run on 6% polyacrylamide sequencing gels containing 8 M urea. The gels were dried, autoradiographed for 16–24 h and scored for LOH. Multiple exposures were used before scoring for LOH. Allelic imbalance indicative of LOH as scored when there was more than 50% loss of intensity of one allele in the tumor sample with respect to the matched allele from normal tissue.

Induction of HtrA1 expression by 5-aza-2' deoxycytidine

The ovarian cancer cell line OV207 was treated with various concentration of 5-aza-2' deoxycytidine (Sigma, St Louis, MO, USA) for 3 days, with addition of fresh 5-aza-2' deoxycytidine every day, and HtrA1 expression was analysed by RT-PCR.

Mutational analysis by DHPLC

Samples used for mutation screening and sequencing were amplified in 20 µl reaction volumes containing 50 ng of genomic DNA, 25 pmoles each of sense and antisense primers, dNTPs (Perkin-Elmer, Foster City, CA, USA), 0.2 ml of *Taq polymerase* (AmpliAq Gold, Perkin-Elmer), 1 × buffer

provided by the manufacturer, and 1.5 mM of MgCl₂. PCR amplification was for 30 cycles using the following profile: 94°C for 30 s, the optimized annealing temperature for 30 s, and 72°C for 30 s. The enzyme was initially activated by denaturation at 95°C for 9 min, and final extension was performed at 72°C for 10 min. The annealing temperatures for various primer sets were 56°C for HtrA1 exons 2, 4, 6, 7, 8, and 9; 58°C for exon 3; 52°C for exon 5. Exon 1 was amplified using GC melt (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions. The primers used for DHPLC analysis are listed in Table 2.

Establishment of antisense HtrA1 stable transfectants

Exponentially growing SKOV3 cells in 100 mm dishes were washed with serum-free medium, and incubated with a mixture of 5 µg of plasmid, 30 µl of LipofectAmine, and 20 µl of Plus reagent (Invitrogen, Carlsbad, CA, USA). After 3 h incubation, complete medium with serum was added. Beginning 24 h after the start of transfection, G418 was added to a final concentration of 400 µg/ml to select the transfectants. For controls, cells were similarly transfected with empty pcDNA3.1+ vector and selected.

Soft-agar assay

Complete medium containing 1% low-melting point temperature agarose was poured into six-well plates (2 ml per well) and allowed to solidify at 4°C to form a bottom layer. SKOV cells (5000/well; vector or antisense transfected clones) were mixed in complete medium with 0.5% agarose and seeded as a top layer. The agarose was solidified at 4°C and then incubated at 37°C. On day 16, the colonies were stained with 1 ml of PBS containing 0.5 mg/ml *p*-iodonitrotetrazolium violet, which is converted into colored product by live cells only. Micrographs were taken at 10× using a Spot II-RT digital camera (Nikon, Millburn, NJ, USA), and colonies larger than 50 and 100 µm in diameter were counted.

Electroporation

At 2 days prior to transfection, cells were incubated in antibiotic-free culture medium. On the day of transfection, two million cells in 0.4 ml Cytomix (van den Hoff *et al.*, 1992) were mixed with 20 µg DNA, and electroporated in 0.4 cm cuvettes using BTX T820 square wave electroporator (BTX, San Diego, CA, USA). Typical settings for electroporation were two pulses of 5 ms duration at 330 V for OV202, resulting in 86% transfection efficiency. Immediately after electroporation, cells

were allowed to recover for 10 min at room temperature before plating in antibiotic-free culture medium.

Assessment of cell death

At 24 h after the electroporation, free-floating cells in the medium were collected, stained with trypan blue, and counted in a hemacytometer. Approximately 20% of cells died from electroporation. The amount of cell death in mock electroporation served as a baseline for determining % cell death in S328A- or HtrA1-transfected groups.

Annexin V labeling and flow cytometry

Annexin V-PE (PharMingen, San Diego, CA, USA) labeling was performed according to the supplier's instruction. Briefly, cells transfected overnight with wild-type or protease mutant HtrA1 were released by trypsinization and sedimented at 200 g for 5 min. All further steps were performed at 4°C unless otherwise indicated. Samples were washed two times with cold PBS and resuspended in 1× binding buffer at a concentration of 1 × 10⁶ cells/ml. Then, 100 µl of solution was transferred to a new 5 ml culture tube, and 5 µl of Annexin V-PE and 7-AAD were added. Cells were incubated for 15 min at 25°C in the dark. In all, 400 µl of 1× binding buffer was added into each tube before flow microfluorimetry on a Becton-Dickinson FACScan (San Jose, CA, USA). Annexin V-stained cells were analysed by FlowJo software (Ashland, OR, USA).

Abbreviations

DHPLC, denaturing high-performance liquid chromatography; IGFBP, insulin-like growth factor binding protein; PDZ, postsynaptic density protein 95-Discs large-Zona occludens 1; LOH, loss of heterozygosity; OSE, ovarian surface epithelium; XIAP, X-linked inhibitor of apoptosis protein; H&E, hematoxylin and eosin; zVAD(OMe)-fmk, *N*-(*N*'-benzyloxycarbonylvalinylalanyl) aspartic acid (*O*-methyl ester) fluoromethylketone; PE, phycoerythrin; 7-AAD, 7-amino-actinomycin.

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