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Genetics

Activation of the human homologue of the *Drosophila sina* gene in apoptosis and tumor suppression

(development/zinc finger protein/RNA-binding protein/H-1 parvovirus/chromosome 16q)

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ABSTRACT **Developmentally regulated genes in *Drosophila*, which are conserved through evolution, are potential candidates for key functions in biological processes such as cell cycle, programmed cell death, and cancer. We report cloning and characterization of the human homologue of the *Drosophila* seven in absentia gene (HUMSIAH), which codes for a 282 amino acids putative zinc finger protein. HUMSIAH is localized on human chromosome 16q12-q13. This gene is activated during the physiological program of cell death in the intestinal epithelium. Moreover, human cancer-derived cells selected for suppression of their tumorigenic phenotype exhibit constitutively elevated levels of HUMSIAH mRNA. A similar pattern of expression is also displayed by the p21^{waf1}. These results suggest that mammalian seven in absentia gene, which is a target for activation by p53, may play a role in apoptosis and tumor suppression.**

Seven in absentia (sina) has been characterized as a gene involved in the fate of the R7 photoreceptor cells during eye development in *Drosophila* (1). The mutant phenotype of sina is not restricted to the eye. Besides other adult sensory organs affected, a 10-fold decrease in lifespan and infertility both in males and in females has been observed (1). Its function is related to the correct integration of signal transduction downstream of the tyrosine kinase receptor sevenless and Ras-1 (2, 3). Its murine homologues (mmsiah1 and mmsiah2) are widely expressed during fetal development as well in the adult (4, 5). The expression of mmsiah2 is increased more specifically during development of the olfactory epithelium, retina, forebrain, and proliferating cartilage. Increased levels of mRNA are also found in a specific population of germ cells within both the mouse ovary and testis (5). Using differential cDNA display (6) we identified mmsiah1b (or TSAP3 for tumor suppressor-activated pathway) among a series of genes induced in the early onset of programmed cell death in a functional model based on wild-type p53-induced apoptosis (7) in murine M1 myeloid leukemia cells (8). In the present study, we asked whether the human homologue of the *Drosophila* gene was implicated in the physiological program of cell death and tumor suppression. Our results suggest that this gene is associated with apoptosis, and, like p21^{waf1}, its expression is activated by both p53- dependent and p53-independent pathways (9–13). More specifically, we investigated the fate of HUMSIAH in human models of tumor suppression developed previously (14) and further expanded here.

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MATERIALS AND METHODS

cDNA Cloning. A human cDNA clone (1.1 kb) was isolated from a cerebellum library (CLONTECH) using as a probe the murine clone TSAP3 (7). The full-length HUMSIAH cDNA was amplified using the Marathon cDNA Amplification Kit (CLONTECH) following the manufacturer's instructions. The antisense primer used to amplify 1.6 kb of the 5′ segment of the transcript is as follows: 5′-CTGGCACACACTCCCACG-CAA-3′. The amplified product was cloned using the TA cloning system (Invitrogen) following the manufacturer's instructions.

Fluorescent *in Situ* Hybridization (FISH). FISH was performed using eight different yeast artificial chromosomes (YACs) as previously described (15). The CEPH YAC library was screened using a PCR procedure (16, 17).

Northern Blot Analysis. Northern blots were performed using 2 μg of poly(A)$^+$ RNA as previously described (7, 18). The entire WAF-1 cDNA (2.1 kb) was used as a probe. For the HUMSIAH, a 1.4-kb fragment of the cDNA was used. Northern blots were hybridized with random primed ^{32}P-labeled probes.

Ultrasensitive Terminal Deoxinucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay and cRNA *in Situ* Hybridization. Multiple confocal three-dimensional imaging was performed on frozen sections (8-μm thick) of the small intestine. For detection of apoptotic cells *in situ* with the TUNEL technique (19), fragmented DNA ends within apoptotic cells were end-labeled with biotinylated poly(dU) by terminal deoxinucleotidyltransferase. TUNEL was revealed using avidin-conjugated peroxidase and tyramide-fluorescein isothiocyanate as substrate (20). Analysis was done by confocal scanning laser microscopy (MRC 600 Bio-Rad) in fluorescence mode. Specific HUMSIAH transcripts were detected by reflectance *in situ* hybridization (RISH) (21), using a sense-antisense system (probe size 230 pb). Hybrids were revealed by antidigoxigenin gold-labeled antibodies. Detection was performed by confocal scanning laser microscopy in reflection mode (MRC 600 Bio-Rad). Pixel calibration was 5.4 pixels per mm.

Abbreviations: HUMSIAH, human seven in absentia homologue; FISH, fluorescense *in situ* hybridization; TUNEL, terminal deoxinucleotidyltransferase-mediated dUTP-biotin nick end labeling; YAC, yeast artificial chromosome.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U63295).

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Selection and Characterization of US3 and US4 Cells. U937 cells were subjected to two rounds of limited dilution to a achieve single clonal population. These cells were infected with H-1 parvovirus as described previously (14). The cytopathic effect of the virus caused massive cell death, sparing two resistant clones US3 and US4 after 3 months of continuous culture. Cell survival is defined as the relative number of the viable cells in H-1 virus infected versus mock-treated culture as measured 4 days after reinfection. To measure tumorigenicity, 10^7 cells of U937, US3, or US4 were injected subcutaneously into *scid/scid* mice (4 to 5 weeks old). Tumorigenicity is expressed as the number of tumors developed within 2 months.

RESULTS AND DISCUSSION

Identification of HUMSIAH. The TSAP3 cDNA (7) was used as a probe to isolate a 1.1-kb fragment from a human cDNA library that was further expanded to the full coding region of the human homologue of seven in absentia (HUMSIAH) by rapid amplification of cDNA ends (RACE)-PCR. Sequence analysis revealed an open reading frame of 846 bp and a 3′ untranslated region extending to 1095 bp. HUMSIAH shares 92.5% identity in its coding nucleotide sequence with mmsiah1b and 67.5% with the *Drosophila* sina gene (data not shown). The amino acid sequence is highly conserved between the human and the *Drosophila* sina proteins (74.5% identity for the homologous regions) and is 98.9% and 96.8% identical to the mmsiah1a and mmsiah1b proteins, respectively (Fig. 1*a*). HUMSIAH encodes a protein of 282 amino acids with a single putative C_3HC_4 RING zinc-finger motif identical to that of the murine and the *Drosophila* proteins (Fig. 1*a*). Recent structural studies of the Wilms tumor protein (WT1) revealed an RNA recognition motif (22, 23). We identified in the HUMSIAH protein conserved domains resembling those described for RNA recognition. As suggested for the Wilms tumor protein, these might be a novel potential mechanism for tumor suppression (22). HUMSIAH encodes a 2.3-kb mRNA expressed in all different tissues tested; an additional transcript of 2.5 kb is present in the placenta (Fig. 1*b*), suggesting an alternative splicing. Such alternative splicing might potentially be an important mechanism involved in tumor suppression. However, this 2.5-kb mRNA was also found on Northern blots using total RNA, suggesting the presence of a pre-mRNA. Specific primers of this gene were used to screen the CEPH YAC (16, 17) libraries. Eight YACs (69G5, 340 kb; 155B9, 440 kb; 159C5, 340 kb; 218E6, 600 kb; 452B7, 310 kb; 464D3, 420 kb; 465B8, 380 kb; 620C9, 640 kb) were isolated. FISH using these YACs revealed a unique localization on chromosome bands 16q12-q13 for HUMSIAH that was further confirmed by using a 1.4-kb cDNA probe (Fig. 1*c*). This region has been reported to contain candidate tumor suppressor genes in various cancers, including breast cancer (24) and Wilms tumor (25–27).

HUMSIAH and Physiological Apoptosis. We further investigated activation of the HUMSIAH gene in a well-established example of physiological programmed cell death. The epithelium of the small intestine has a high turnover rate with a typical kinetics related to its columnar architecture. As the cells migrate from the lower part of the crypt up the villus toward the lumen, they differentiate and finally die by apoptosis at the tips of the villi (19). *In situ* hybridization of intestinal epithelium cells with a HUMSIAH cRNA probe combined with a TUNEL assay indicates activation of this gene

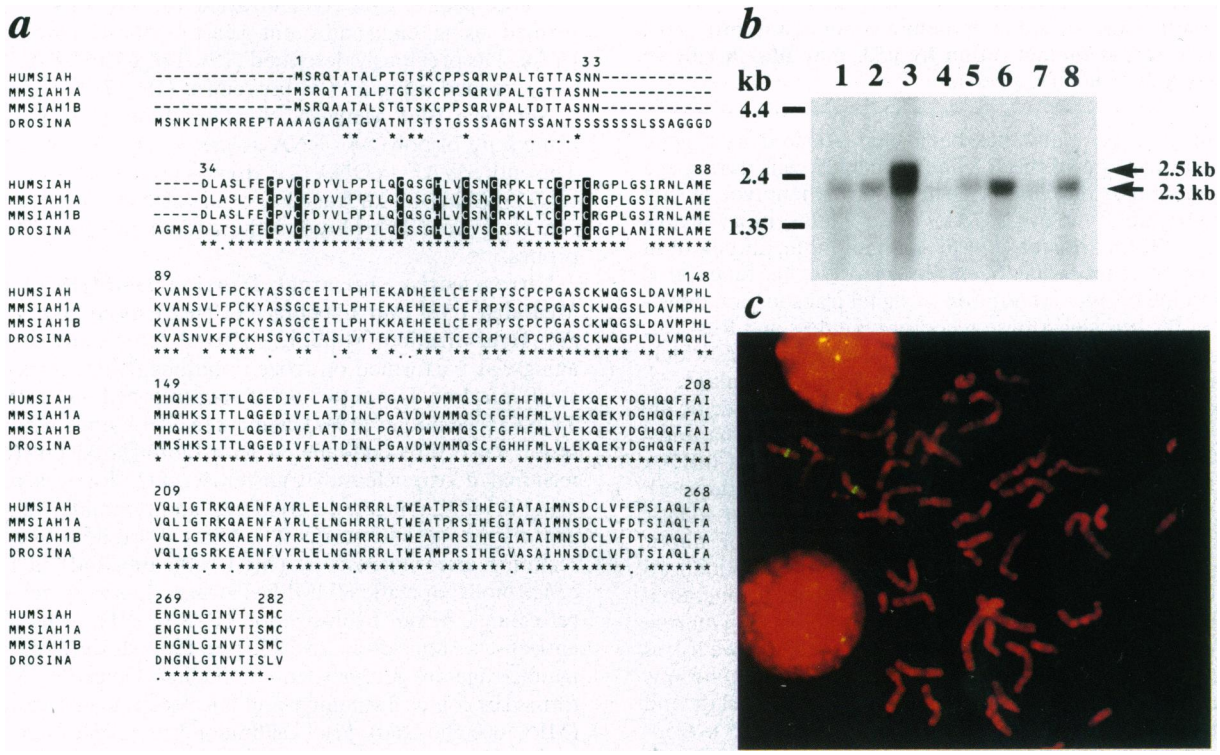

FIG. 1. Cloning, expression, and chromosomal localization of HUMSIAH. (*a*) Amino acid sequence (one letter code) encoded by the HUMSIAH gene and its comparison with the murine (MMSIAH1B) and the *Drosophila* proteins (DROSINA). The human protein of 282 amino acids shows 74.5% identity (for the homologous regions) with the *Drosophila* protein and is 98.9% and 96.8% identical to the mmsiah1a and mmsiah1b proteins, respectively. The unique zinc finger (white letter on a black background) is of C_3HC_4 type and is identical to that of the murine and the *Drosophila* proteins. *, Conservative substitutions; ·, nonconservative substitutions. (*b*) Tissue distribution of HUMSIAH mRNA. Multiple tissue Northern blot (Clontech) was hybridized with a probe (900 bp, from the 3′ untranslated region) as previously described (18). A 2.3-kb transcript is detected in the eight different tissues. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. An additional 2.5-kb transcript is detected in the placenta. (*c*) FISH using YAC 69G5. A unique signal is detected on chromosome 16q corresponding to bands q12-q13.

in these apoptotic cells (Fig. 2). Double labeling (data not shown) experiments confirm that the TUNEL positive cells were also the HUMSIAH positive cells.

Models of Tumor Suppression and Activation of HUMSIAH. To investigate the implication of HUMSIAH in tumor suppression, we used a previously described biological model (14) that uses a global-oriented rather than a single gene-oriented approach to study tumor suppression. From clonal populations of malignant cells, we derived subclones with a suppressed tumorigenic phenotype. This selection took advantage of the H-1 parvovirus, which kills preferentially tumor cells while sparing their normal counterparts (29–31). Selection of cells resistant to the cytopathic effect of the H-1 parvovirus out of a sensitive tumor can give rise to cells with a reduced malignant phenotype (14).

From a clonal population of erythroid chronic myelogenic leukemia K562 cells, we selected KS cells that are resistant to the cytopathic effect of H-1 parvovirus and have a suppressed malignant phenotype both *in vitro* and *in vivo* (14). Whereas the parental K562 cells do not express p53, the KS cells reexpress a wild-type p53 (14). As shown in Fig. 3, the expression of HUMSIAH is constitutively elevated in KS cells, as is also the expression of the $p21^{waf-1}$ gene.

Using the same approach, we isolated from a clonal population of U937 cells, sensitive to the cytopathic effect of H-1 parvovirus, clones US3 and US4 that are resistant to the virus (Table 1). The US3 and US4 clones have a strongly suppressed tumorigenic phenotype (Table 1). While the parental U937 cells develop tumors in 80% of the cases injected into *scid/scid* mice, the US3 cells did not form a single tumor and US4 cells developed one tumor on 20 inoculations with 10^7 cells. As for the previous observation made on KS cells (14), the US3 and US4 clones release infectious H-1 parvovirus even 1 year after the selection (data not shown). Screening for 18 cell surface markers (DR, CD19/B4, CD7/Leu9, CD5/Leu1, CD2/T11, CD38/T10, CD71/T9, CD8/IOT8a, CD4/Leu3a, CD25/IOT14, CD33/My9, CD13/My7, CD14/My4, CD11b/C3Bi, CD36/OKM5, CD34/IOM34, class I/W6.32, CD15/ION1) indicates that there was no shift in differentiation between the U937 cells and US3 and US4 cells (data not shown), suggesting that the change in malignant phenotype was not due to terminal differentiation. U937 cells do not express p53 (data not shown). In contrast to the KS clones, the US3 and the US4 clones do not reexpress p53 (data not shown). However, both US3 and US4 cells display a concomitant activation of WAF-1 and HUMSIAH (Fig. 3). Comparison of U937, US3, and US4 in a TUNEL assay shows that the fraction of apoptotic cells is significantly increased in US3 and US4 cells (Fig. 3 *g–i*). *In situ*

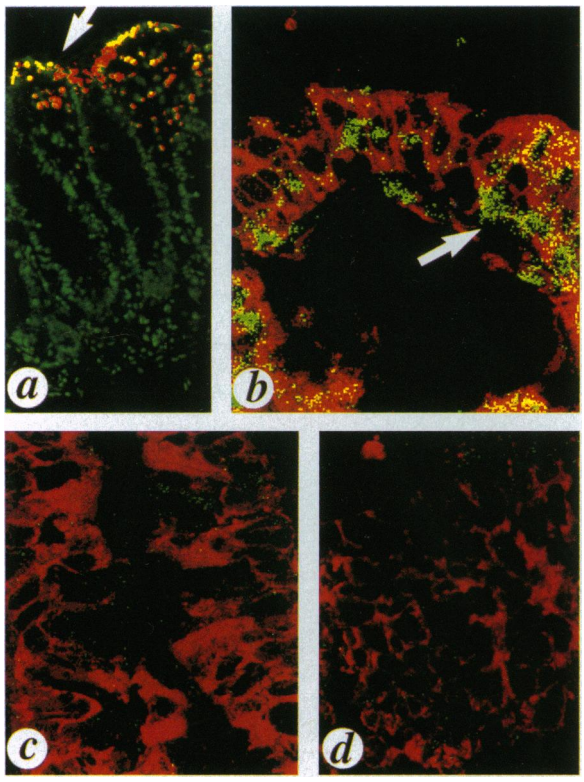

FIG. 2. Physiological expression of HUMSIAH in the apoptotic cells of the intestinal epithelium. (*a*) Section of the intestinal epithelium with its columnar architecture (DNA stained in CA3, green) (×120). Apoptotic cells (yellow, reddish, see arrow) were detected *in situ* with the TUNEL technique (19) at the tip of the intestinal villi. (*b–d*) *In situ* hybridization of the intestinal epithelium (endoplasmatic reticulum stained in $DiOC_6$, red; ref. 28) with a HUMSIAH cRNA probe (green). The apoptotic cells at the tip of the villi (*b*) show an accumulation of the HUMSIAH transcript (green, arrow). This signal is strongly decreased toward the middle section (*c*) and is absent at the bottom (*d*) of the villi.

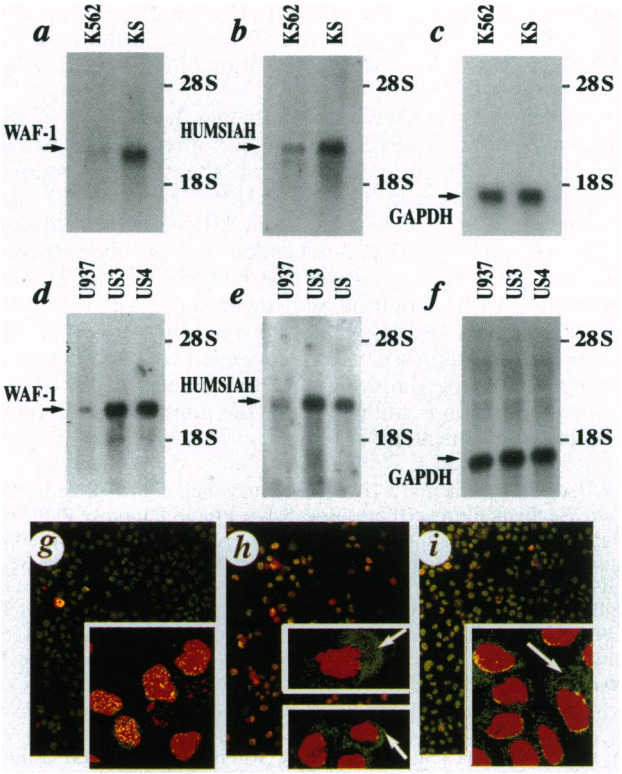

FIG. 3. Activation of HUMSIAH in tumor suppression and apoptosis. Northern blot analysis (*a* and *b*) of WAF-1 and HUMSIAH expression in the parental K562 and the KS cells indicates activation of both genes in the latter. Expression of WAF-1 and HUMSIAH is also activated in the US3 and the US4 cells as compared with the parental U937 cells (*d* and *e*). (*c* and *f*) The GAPDH control. TUNEL (yellow, reddish) assay indicates a strongly activated program of cell death in US3 (*h*) and US4 (*i*) as compared with parental U937 cells (*g*). (*g–i Insets*) *In situ* hybridization with HUMSIAH cRNA shows a marked increase in transcript levels (green, arrow) in US3 and US4 committed to apoptosis. (*Insets*) DNA is labeled with CA3 red.

Table 1. Resistance to H-1 parvovirus and tumorigenicity of U937, US3, and US4 cells

Cell lines	Cell survival to H-1 virus infection multiplicity of infection 100% of mock-treated cells	Tumorigenicity *scid/scid* mice
U937	0.4	16/20
US3	96	0/10
US4	89	1/20

hybridization with the HUMSIAH cRNA indicated that the US3 and US4 cells committed to apoptosis and positive in the TUNEL assay, exhibit a strong activation of HUMSIAH (Fig. 3 *g–i Insets*).

In conclusion, we show that the human homologue of the sina gene, highly conserved through evolution, is expressed at elevated levels under conditions of apoptosis and tumor suppression. Moreover, like the $p21^{waf-1}$ gene (9–13) that encodes a cell cycle inhibitory protein, HUMSIAH expression is also activated by both p53-dependent and p53-independent pathways. This findings suggest that HUMSIAH may play an important role in apoptosis and tumor suppression. Finally, our experiments demonstrate that no transfer of specific human tumor suppressor genes is needed to achieve a suppressed phenotype, implying that the genetic potential for tumor suppression is still present in the tumor cells, although it needs to be activated.

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