Expression of a Novel Human Gene, Human Wings Apart-Like (hWAPL), Is Associated with Cervical Carcinogenesis and Tumor Progression

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ABSTRACT

In Drosophila melanogaster, the wings apart-like (wapl) gene encodes a protein that regulates heterochromatin structure (1). Mutations of wapl prevent the normal close apposition of sister chromatids in heterochromatin regions but do not appear to affect either heterochromatin condensation or chromosomal segregation (1). This evidence suggests that wapl is required to hold sister chromatids together in mitotic heterochromatin. Wapl has also been implicated in both heterochromatin pairing during female meiosis and the modulation of position effect variegation (1). In addition, a P element screen of Drosophila identified wapl as a modifier of chromosome inheritance (2).

Among all varieties of cancer, uterine cervical cancer is unique because of its association with high-risk human papillomavirus (HPV) infection, with strains like HPV-16 and HPV-18. High-risk HPV encodes two oncoproteins, E6 and E7, which subvert crucial cellular growth, and the host cell. E6 accelerates proteosomal degradation of the p53 tumor suppressor, and E7 inactivates the retinoblastoma protein, interfering with the action of both p16INK4a (3) and the cyclin-dependent kinase inhibitor p21(Cip1) (4, 5). Both the E6 and E7 high-risk HPV oncoproteins independently induce genomic instability in normal human cells (6, 7). Only a small portion of precursor lesions infected with HPV, however, develops into invasive carcinomas (8). Therefore, additional genetic and microenvironmental factors subsequent to HPV infection are thought to play an important role in the initiation and progression of cervical neoplasia (8–10).

In this study, we describe the isolation and characterization of a novel human wapl-related gene termed human WAPL (hWAPL). We have also demonstrated that hWAPL has the characteristics of an oncogene and is associated with uterine cervical cancer.

INTRODUCTION

The wings apart-like (wapl) gene of Drosophila melanogaster encodes a protein that regulates heterochromatin structure (1). Variations in wapl prevent the normal close apposition of sister chromatids in regions homologous to heterochromatin, but do not appear to affect either the heterochromatin condensation or chromosomal separation (1). This evidence suggests that wapl is required to hold sister chromatids together in mitotic heterochromatin. Wapl has also been implicated in both heterochromatin pairing during female meiosis and the modulation of position effect variegation (1). In addition, a P element screen of Drosophila identified wapl as a modifier of chromosome inheritance (2).

Material and Methods

Cloning and Construction of the hWAPL Expression Vector. To isolate the complete hWAPL cDNA sequence, we used a human testis Marathon-Ready cDNA kit (Clontech, Palo Alto, CA).

First-strand cDNA synthesis was performed as described (13). Real-time PCR analysis was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) with SYBR Green I (Cambrex, Washington, DC). Real-time PCR used the hWAPL-specific primers 5′-GAATTCTAGACAGCGCTGA-ACGTGTGTG-3′ and 5′-TTGAAATCTCTAATGTTCTCAAATATTTCA-ATCACACCTAGG-3′ and inserted into the hemagglutinin (HA)-tagged mammalian expression vector, pHM6 (HA-hWAPL; Roche Diagnostics, Mannheim, Germany).

Immunohistochemistry and Immunoblot Analysis. To generate mouse monoclonal antibodies against hWAPL, we immunized mice against a 6×histidine-tagged hWAPL COOH terminus (amino acids 814–1037) fusion protein. Spleen cells of an immunized mouse were fused with P3UI mouse myeloma cells as described previously (14). Of the 128 hybrids generated, one clone (clone R929) showed exclusive reactivity with hWAPL by ELISA. We used the supernatant of this clone as anti-hWAPL antibody.

Animals and Treatment. BALB/cA-Jcl-nu female mice (4 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan).

The tumorogeneity of the stable NIH3T3 transformants overexpressing hWAPL in vivo was examined as described previously (16). The anti-HPA (Roche Diagnostics; 3F10) and monoclonal anti-tubulin clone B-5-1-2 (Sigma Chemical Co., St. Louis, MO; T-5168) antibodies were purchased.

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generated siRNAs using a Silencer siRNA Construction Kit (Ambion, Austin, TX). siRNA transfection was performed in DMEM without serum using Oligofectamine Reagent (Invitrogen Japan, Tokyo, Japan) and Opti-MEM I (Invitrogen Japan).

For cell quantitation, we harvested the cells from the wells of a 12-well plate and resuspended them in 100 μl of PBS. Trypan blue solution (100 μl, 0.4%; Sigma) was added to each sample, and viable cell numbers were quantitated using an erythrometer. The results shown are representative of three independent cell count analyses.

RESULTS

Molecular Cloning of hWAPL. To isolate wapl-related genes from human cells, we searched DNA databases and identified a cDNA fragment, KIAA0261 (17), and three expressed sequence tag clones, BE410177, BF79516, and BE257022, containing the KIAA0261 sequence. We also performed 5' rapid amplification of cDNA ends. From these DNA sequences, we cloned and confirmed the full-length coding region sequence of the cDNA containing KIAA0261. We named this gene hWAPL (GenBank accession no. AB065003) to reflect its homology to wapl. The hWAPL gene product shows high sequence similarity in the WAPL-conserved region (amino acids 627-1169, 34% identical and 56% similar) and low similarity throughout the other regions to the wapl gene product. Several additional stretches of amino acids are also present in wapl protein (Fig. 1A).

High-Level Expression of hWAPL in Human Cervical Cancer. As wapl is involved in sister chromatid cohesion, hWAPL may modify chromosomal inheritance. Deregelation of the expression of genes involved in chromosomal inheritance directly induces a variety of disorders associated with aneuploidy, including birth defects and cancer. Northern blot analysis detected hWAPL mRNA expression in several invasive cervical cancer samples, examined in tandem with additional human cancers and normal tissues (Fig. 1B). We confirmed the hWAPL expression in cervical cancers by quantitative real-time PCR analysis of tumor and normal tissue samples. The levels of hWAPL mRNA expression in cervical cancers were significantly higher than the levels observed in either normal cervical controls or endometrial, ovarian, breast, lung, stomach, renal, and colon cancers (Fig. 1C).

To investigate the connection between hWAPL expression and oncogenesis in cervical malignancies, we examined the expression of hWAPL by immunohistochemistry in a series of clinical samples of the various grades of cervical dysplasia [cervical intraepithelial neoplasia (CIN) I-III] and invasive squamous cell carcinoma. We found nuclear immunostaining for hWAPL in all samples (Fig. 2A). hWAPL expression in benign squamous epithelia was confined to the basal and parabasal cell layers. In contrast, hWAPL expression in squamous dysplasia and invasive carcinoma increasingly appeared in the more superficial cell layers and was significantly increased compared with the adjacent benign epithelia (P = 0.0002 for CIN I, P = 0.0003 for CIN II, P = 0.0001 for CIN III, and P = 0.0001 for invasive squamous cell carcinoma; Wilcoxon’s signed rank test). CIN I and II cases showed hWAPL expression in the basal 50 and 70% of the epithelial thickness, respectively, whereas CIN III and invasive squamous cell carcinoma showed hWAPL expression in the full thickness of the dysplastic epithelia (Fig. 2A). Furthermore, the mean hWAPL staining score increased remarkably with increasing grade of dysplasia (Fig. 2B). These data strongly suggest that the unscheduled high-level expression of hWAPL may play a significant role in cervical carcinogenesis and tumor progression.

hWAPL Has Oncogenic Characteristics. Because we observed high-level expression of hWAPL in tumors, we sought to determine whether hWAPL overexpression promotes tumor development. We transfected NIH3T3 cells with an HA-tagged hWAPL expression vector (HA-hWAPL 3T3) or HA expression vector (HA-3T3). Then, we compared the ability of HA-hWAPL 3T3 with HA-3T3 cells to grow as tumors in nude mice. We injected 10^6 cells into three s.c. sites of each nude mouse. HA-hWAPL 3T3 cells produced tumors in all nude mice within 10 days after injection of cells (100%, n = 18; Fig. 3A). HA-3T3 failed to produce tumors in any mice (0%, n = 18). We confirmed high hWAPL expression levels in the resultant tumors by Western blot analysis (Fig. 3B). These results suggest that hWAPL has the characteristics of an oncogene.

Repression of hWAPL Expression Induces Cell Death. We examined hWAPL function by suppressing hWAPL expression. Initial attempts to generate a WAPL-deficient mouse demonstrated that the loss of WAPL was embryonic lethal (data not shown). Therefore, we designed two 21-nucleotide, double-stranded siRNAs, siRNA(I) and siRNA(II), to repress hWAPL expression (Refs. 18 and 19; Figs. 1A

Fig. 1. Structures of wings apart-like (WAPL) proteins and human WAPL (hWAPL) expression in normal and tumor human tissues. A, schematic structure of the hWAPL and Drosophila wap1 gene products. The site corresponding to the probe sequence used for Northern blot analysis is indicated by “probe.” The antibody recognition site is indicated by “hWAPL-C.” The small interfering RNA (siRNA) targeting sites are indicated by “siRNA(I)” and “siRNA(II).” B, Northern blot analysis of hWAPL in several normal (N) and tumor (T) human tissues. C, quantitative real-time PCR analysis demonstrating hWAPL mRNA levels in various normal (N) and tumor (T) human tissues. Columns, the means of examined samples. The minimum mRNA expression level was arbitrarily set to 1 in the graphical presentation; all other mRNA signals were normalized to this value. Bars, SD.
We examined various human cancer-derived cell lines and found that cervical cancer-derived cell lines containing both HPV-positive and -negative cells exhibited higher levels of hWAPL expression compared with the other cell lines (data not shown). Then, we examined the effects of suppressing hWAPL in a cervical cancer-derived cell line, SiHa. siRNA transfection at a concentration of either 1 nM siRNA(I) or siRNA(II) reduced hWAPL mRNA levels (Fig. 4B). siRNA(I) was more effective at reducing hWAPL mRNA than siRNA(II). Thus, we used siRNA(I) in the subsequent experiments. hWAPL protein levels were also significantly reduced after siRNA(I) transfection (Fig. 4C). Interestingly, siRNA(I) repressed the growth of the cells and subsequently induced cell death (Fig. 4, D and E). siRNA(II) repressed cell growth in a similar manner as siRNA(I) (Fig. 4D), suggesting that the effects of these siRNAs on proliferation and viability are likely caused by the repression of hWAPL expression. Similar results were obtained in another cervical cancer-derived cell line, CaSki, with 10 nM siRNA(I) (data not shown). On the contrary, we did not observe any effects of siRNA(I) on cells expressing relatively low levels of hWAPL, such as Saos-2 and HCT116 (data not shown).

To investigate the fate of cells transfected with siRNA(I), we analyzed siRNA-transfected cells by flow cytometry (Fig. 5). In siRNA(I)-transfected cells, the population of cells exhibiting S phase DNA content increased (Fig. 5, 48 and 72 h). In addition, there was an increase in the number of apoptotic cells exhibiting subG1 DNA content (Fig. 5; 72 h). Many cells showing S phase DNA content may also be apoptotic cells at G2-M phase. Taken together, these results suggest that a malfunction in the hWAPL pathway activates an S phase checkpoint or another apoptotic pathway and consequently leads to cell death.

DISCUSSION

In this study, we report the isolation and characterization of a novel human gene termed hWAPL. We were unable to identify additional genes similar to wapl within the human genome sequence database. Thus, although the high-sequence conservation between hWAPL and wapl is limited to a third of the protein sequence encoded by wapl (Fig. 1A), we consider hWAPL to be the human homologue of wapl. We did not find any protein sequence motifs in hWAPL, except for the WAPL-conserved region (Fig. 1A). We therefore expect that hWAPL has similar functions to the wapl protein. Two hybridization signals for hWAPL were visible by Northern blot analysis (Fig. 1B). Western blot analysis, however, detected only a single band for
hWAPL (Fig. 2C). In addition, we did not obtain additional nucleotide sequences similar to the open reading frame of hWAPL by PCR analysis with various PCR primers (data not shown). Thus, we consider that the two hybridization signals may reflect the difference of the length of the untranslated regions of the hWAPL mRNA.

High-level expression of hWAPL was observed in cervical cancers (Fig. 1, B and C). Furthermore, hWAPL-overexpressing 3T3 cells developed into tumors on injection into nude mice (Fig. 3). These results suggest that hWAPL has oncogenic characteristics. Cervical cancer is a serious health problem, with ~500,000 women developing the disease each year worldwide. In many developing countries, it is the most common cause of cancer death and years of life lost because of cancer (20). Although the fundamental role of high-risk HPV infection in the pathogenesis of cervical carcinoma is well established, other factors are thought to play a role in cervical carcinogenesis (8, 21). Because all of uterine cervical samples examined were HPV positive (data not shown), it is still to be confirmed whether hWAPL expression is inducible by HPV infection. However, HPV-positive normal cervical tissue samples exhibited low hWAPL expression (Fig. 1, B and C and data not shown), and an HPV-negative, uterine cervical cancer-derived cell line, C33A, showed high hWAPL expression (data not shown). Thus, hWAPL expression is likely to be more closely related with cervical carcinogenesis than HPV infection. Recently, Acs et al. (9) found significant correlation among expression of Epo receptor, p16INK4a, and bcl-2 in benign and dysplastic squamous epithelia. In our results, hWAPL showed similar expression pattern to Epo receptor and p16INK4a in benign and dysplastic cervical squamous epithelia and invasive squamous cell carcinomas (Fig. 2, A and B). Although we did not find any evidence for hWAPL being involved in hypoxia-inducible Epo signaling, hWAPL may cooperate with the Epo signaling in the progression of cervical neoplasia. These observations indicate that hWAPL overexpression can be used as a useful diagnostic tool in the detection of cervical dysplasia like p16INK4a (22) and Epo receptor (9). In addition, our results provide the necessity to investigate the potential of hWAPL as a cancer therapeutic target.

Fig. 4. Repression of human wings apart-like (hWAPL) expression by small interfering RNA (siRNA) treatment induces cell death. A, sequences and structures of siRNAs. The negative control siRNA possesses the same nucleotide composition as siRNA(I) but lacks homology to any known human genes. B, reduction of the hWAPL transcript by siRNA in SiHa cells. After siRNA transfection, SiHa cells were harvested at either 48 or 72 h. Total RNA was extracted from the cells and subjected to real-time PCR analysis. I, siRNA(I); II, siRNA(II); nc, negative control siRNA; wt, untransfected wild type. Data were normalized to a maximum mRNA level that was arbitrarily set to 1 in the graphical presentation. C, reduction of hWAPL protein levels by siRNA. Western blot analysis of total cell extracts from untreated SiHa or SiHa cells 72 h after transfection with siRNA(I) or negative control siRNA. α-tubulin is shown as a loading control. D, active siRNA specific for hWAPL induces cell death. SiHa cells transfected with siRNA(I), siRNA(II), or negative control siRNA were harvested at 24, 48, 72, and 96 h after transfection. Cell numbers were counted using an erythrometer. Bars, SE. E, representative phase-contrast images of SiHa cells transfected with siRNA(I) and negative control siRNA are shown.
the other samples. Iodide and subjected to flow cytometric analysis to examine DNA content. A total of then harvested at 24, 48, and 72 h after transfection. Cells were stained with propidium transfection. SiHa cells were transfected with either siRNA(I) or negative control siRNA, alated that the novel oncogene, hWAPL in normal and malignant cells, our results have demon-
matin organization (1). Thus, we expect that hWAPL is also associ-
In addition, hWAPL may cause apoptosis and/or arrest of cells at S phase (Fig. 5).

Loss of WAPL was embryonic lethal in mouse (data not shown), and repression of hWAPL expression in SiHa cells led to cell death (Fig. 4). Flow cytometry analysis demonstrated that malfunction of hWAPL may cause apoptosis and/or arrest of cells at S phase (Fig. 5). In addition, *Drosophila wapl* is associated with regulation of chromatin organization (1). Thus, we expect that hWAPL is also associated with regulation of chromatin structure, and deregulation of hWAPL expression may induce chromosomal instability. Although additional investigations are necessary to elucidate the actual function of hWAPL in normal and malignant cells, our results have demonstrated that the novel oncogene, *hWAPL*, is one of the essential genes for development and cell growth and may play a significant role for cervical carcinogenesis and tumor progression.

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![Figure 5. Flow cytometric analysis of SiHa cells after small interfering RNA (siRNA) transfection. SiHa cells were transfected with either siRNA(I) or negative control siRNA, then harvested at 24, 48, and 72 h after transfection. Cells were stained with propidium iodide and subjected to flow cytometric analysis to examine DNA content. A total of 50,000 cells was counted for the sample siRNA(I) 72 h, and 20,000 cells were counted for the other samples.](image-url)
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