RESEARCH ARTICLE

Expression of hPOT1 in HeLa Cells and the Probability of Gene Variation of *hpot1* Exon14 in Endometrial Cancer are Much Higher than in Other Cancers

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Abstract

To investigate the expression of hPOT1 in the HeLa cell line and screen point mutations of *hpot1* in different tumor tissues a two step osmotic method was used to extract nuclear proteins. EMSA was performed to determine the expression of hPOT1 in the HeLa cell line. PCR was also employed to amplify the exon14 sequence of the *hpot1* gene in various of cancer tissues. A SV gel and PCR clean-up system was performed to enrich PCR products. DNAStar was used to analyse the exon14 sequence of the *hpot1* gene. hPOT1 was expressed in the HeLa cell line and the signal was gradually enhanced as the amount of extracted nuclear proteins increased. The DNA fragment of exon14 of *hpot1* was successfully amplified in the HeLa cell line and all cancer tissues, point mutations being observed in 2 out of 3 cases of endometrial cancer (66.7%) despite the *hpot1* sequence being highly conserved. However, the sequence of *hpot1* exon14 do not demonstrate point mutations in most cancer tissues. Since hPOT1 was expressed in HeLa cell and the probability of gene point variants was obviously higher in endometrial cancer than other cancers, it may be involved in the pathogenesis of gynecological cancers, especially in cervix and endometrium.

Keywords: *Hpot1* - exon14 - endometrial cancer - point variation - EMSA

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Introduction

Telomeres which protect the ends of linear eukaryotic chromosomes from illegitimate recombination, degradation and recognition as DNA double strand break are constituted of a double-stranded TTAGGG repeats for 2–20 kb and a single stranded overhang for 50–500 nucleotides (de Lange, 2005). The protection of the chromosome ends by telomeres is centrally involved in cell division. For the length of the telomeric sequence declines until the telomeres become critically short as cell division, so normal cells have a limitation of division number. But cancer cells could escape this limitation. Telomeres would be completely repaired by telomerase and related proteins in cancer cells contributes to the phenomenon (Hanahan et al., 2011; Mason et al., 2011), which are mediated by a stably associated complex—shelterin. Shelterin which is composed of six telomere proteins, including TRF1 (telomere repeat binding factor 1), TRF2 (telomere repeat binding factor 2), TIN2 (TRF1-interacting factor 2), TPP1 (POT1 and TIN2-interacting protein), Rap1 (telomeric repeat-binding factor 2-interacting protein 1) and POT1 (protection of telomeres) (de Lange, 2005; Diotti et al., 2011).

hPOT1(human protection of telomeres) shares weak sequence similarity with the N-terminal DNA binding domain of TEBPs from ciliated protozoa and was named POT1 based on the rapid loss of telomeric DNA that occurs following deletion of the gene (Baumann et al., 2001). hPOT1 mainly regulates telomerase-mediated telomere extension (Colgin et al., 2003) and protect telomeres from end-to-end chromosomal fusions (He et al., 2006). In addition, hPOT1 may involved in cell cycle regulation (Wu et al., 2006), apoptosis (Wan et al., 2011) and so on. Many studies had reported that hPOT1 is correlated with a broad range of cancers, for example, gastric cancer (Wan et al., 2011), papillary thyroid cancer (Cantara et al., 2012), breast cancer (Shen et al., 2010), leukemia (Poncet et al., 2008). Some reports centered in gene variations of *hpot1*. Savage, S. A. found some single nucleotide polymorphisms(SNP) of *hpot1* in breast cancer (Savage et al., 2007). Others founded 2 SNPs of *hpot1* are the risk factors of the lung cancer (Choi et al., 2009). There are *hpot1* genetic point variations in the skin cancer (Nan et al., 2011).

hpot1 gene locates on chromosome 7. The whole *hpot1* gene which assemble a genomic sequence of approximately 120 kb contains 22 exons which encode at

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least five splice variants. However, four exons are subject to exon skipping in transcripts. The exon size and the position of splice sites are highly conserved for exons 6 to 20. Variant 2 splice site is between exon 12 and exon 13. Variants 3 and 4 originate by exon skipping of exon 17 and exon 8 (Baumann et al., 2002). So it is worthy to determine the changes from exon 8 to exon 17. However, the expression and exact point mutations of *hpot1* exon14 in cancers tissues remained largely unknown. Here we wanted to determine the expression of hPOT1 in cancer cell and screen exon14 base point mutations of *hpot1* in various cancer tissues.

Materials and Methods

The human cell lines HeLa cell was obtained from our previous research prepared for this study. The cells were cultured in RPMI1640 (HyClone, Utah, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA). All cultures were grown at 37˚C under a humidified atmosphere of 5% carbon dioxide for routine growth.

From January 2005 to January 2006, 20 cases of specimens samples for DNA extracted were obtained from surgically resected many kinds of tumors tissues in the First Affiliated Hospital of Guangdong Medical College. All samples were frozen immediately, stored in liquid nitrogen. The median ages of 20 tumors tissues for DNA extraction was 59 years old (range from 39yr to 74yr). All persons gave their informed consent prior to their inclusion in the study. This study was approved by the ethics committee for gene variations research of the Guangdong Medical College and was conducted according to the Declaration of Helsinki Principles.

Total DNA was extracted with traditional Phenol-Chloroform method. Briefly, the tissues was crushed to pieces in liquid nitrogen. Then transferred to an eppendorf tube and centrifuged for 10 min at 5000 g (4°C). The supernatant was discarded and the pellet was rinsed with 1 ml of cold TE buffer (10 mM Tris-HCl pH 7.5 and 0.1 mM Na_2EDTA), vortexed and centrifuged 10 min at 5000 g (4°C). This step was repeated 2 times. The cellular pellet was resuspended with 1.2 ml of nuclei lyses buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na2EDTA, pH 8.2). The cell lysates were water bathed at 37°C for 3h with 10% SDS and 6 μL of a proteinase K solution (2 mg/ml proteinase K, 1% SDS and 2 mM Na2EDTA). After digestion was complete, samples were treated with 0.5 mL of Tris-HCl (pH 8.0) sature phenol chloroform (1:1) and repeated two times. Finally, the aqueous phase was precipitated with a 1:1/10 volume of 3 M Sodium Acetate (pH 5.2) and a 1:1 volume of 100% isopropanol for 2 min at room tempera -ture. After centrifugation at 11 000 g for 10 min the supernatant was discarder and pellet was washed with 1 ml of 70% ethanol (chilled at -20°C), dried and resuspended with 50μL TE buffer.

Nuclear protein was extracted as described previously (Porquet et al., 2011). Firstly, cells were collected at room temperature by centrifuged 1000 rpm for 5 min. All cells are carried out in a room temperature tubules between steps less than 30 seconds, the samples are placed on ice. The cell suspension is then transferred to a tubule. Cells are pelleted for 10 seconds and resuspended in 400μL cold Buffer A (10 mM HEPES-KOH pH 7.9 at 4°C 1.5 mm $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) by flicking the tube. The cells are allowed to swell on ice for 10 minutes, and then vortexed for 10 seconds. Samples are centrifuged for 10 seconds, and the supernatant fraction is discarded. The pellet is resuspended in 20-100/d (according to starting number of cells) of cold Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris is removed by centrifugation for 5 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at -70°C.

The procedures were performed according to previous publication (Latrick et al., 2010). In brief, HeLa cells nuclear extracts were prepared according to anterior described method Nuclear Protein Extraction for identifying protein-DNA interaction assay. The oligonucleotide probes with synthesized according to human pot1 binding oligonucleotide sequence, was labeled with [γ-32P]-ATP using T4 polynucleotide kinase and purified by chromatography through Probe- QuantTM G-50 micro columns. The oligonucleotide probes sequences: TTAGGGTTAGGGTTAGGGTTAGGG. 5μg of nuclear proteins or 2.5 μg of nuclear extracts were incubated with [γ-32P]-ATP labeled probe (20,000 cpm) at room temperature for 20 min in a total of 20 μL of a reaction mixture containing (final concentrations): 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol and 1 μg of poly (dI-dC) as non-specific competitor. For competition EMSA, 100 pmol or a serial dilution of unlabeled probe was added in the reaction mixture and pre-incubated at room temperature for 10 min to verify the specificity of protein-DNA interactions. The resulting protein-DNA complexes were analyzed by electrophoresis on a 8% polyacrylamide gel followed by autoradiography and densitometry analysis.

DNA primers were designed according to GeneBank. Human *hpot1* Gene(Gene ID: 25913) DNA primers sequence were designed as follows: sense: 5'-GCAAAAGGAGTATTCTAACAAACAG-3' and antisense: 5'-TCACGCTTACACCAAAATCG- 3' for *hpot1* exon14. PCR amplifications were performed on the PTC-200 PCR equipment in a total volume of 25μL. Each reaction contained 12.5μL of the 10×reaction buffer, 0.5μL of dNTPs, 0.1μL of Platinum Taq DNA polymerase, 1μL of $MgSO_4$ (2 mM final concentration), 1μL of each primer (200 pmol/μL), 2μL of gDNA and 18.4μL of RNase free H_2O. The cycling conditions for all the cDNA included pre-incubation for 5 min at 94˚C and followed by 30 cycles of 30 sec at 94˚C, 30 sec at 57˚C, 30 sec at 72˚C and a final extension for 10 min at 72˚C. The products stored in 4˚C. All experiments were repeated at least three times. PCR products were observed using electrophoresis on 1.0% agarose gels containing 0.5% SYBE GreenI. Gels were observed at ultraviolet and took the corresponding images. While the densities of PCR Gels images were

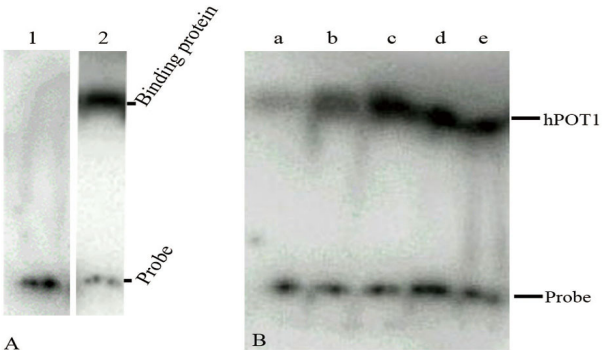

Figure 1. The Expression of hPOT1 Detected by EMSA in the Nuclear Extracts of HeLa Cell. (A) EMSA method' establishment. 1: the negative result, 2: the positive result. (B) EMSA image of hPOT1 expression in given amount total nuclear protein. a: 0.5μg, b: 1μg, c: 2.5μg, d: 5μg, e: positive control

quantified with gel analysis software.

The PCR products were purified using Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI, USA). Briefly following electrophoresis, excised DNA band from gel and placed gel slice in a 1.5 mL microcentrifuge tube.. Added 10μL Membrane Binding Solution per 10mg of gel slice. Vortexed and incubated at 50-65˚C until gel slice was completely dissolved. Added an equal volume of Membrane Binding Solution to the PCR amplification. Insert SV minicolumn into Collection Tube. Transferred dissolved gel mixture or prepared PCR product to the minicolumn assembly. Incubated at room temperature for 1 minute. Centrifuge at 16,000×g for 1 minute. Discarded flow through and reinserted minicolumn into Collection Tube. Added 700μL Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discarded flow through and reinsert minicolumn into Collection Tube, repeated this step with 500μL Membrane Wash Solution. Centrifuged at 16,000×g for 5 minutes. Empty the Collection Tube and recentrifuged the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Carefully transferred minicolumn to a clean 1.5 mL microcentrifuge tube. Added 50μL of nuclease-free water to the minicolumn. Incubated at room temperature for 1 minute. Centrifuged at 16,000 × g for 1 minute. Discard minicolumn and stored DNA at –20°C.

Then the purified PCR products were sequenced at the SanGon Biotech Corp (Shanghai, China). The software DNAStar version 5.0 (Madison, WI, USA) was used for sequence assembly and multiple sequence alignment to confirm either identities or similarities.

Results

Considering EMSA is a effectively method for detecting DNA binding proteins, firstly we established the EMSA method. NF-ϰB protein was used as the positive control of EMSA. Meanwhile, the purified probe was thought as the negative control. The purified probes ran much faster when ran native electrophoresis for probes is a only length of oligonucleotide with small molecular weight (Figure 1 A. lane 1). After probes incubated with NF-ϰB proteins, the probes would bind to NF-ϰB so

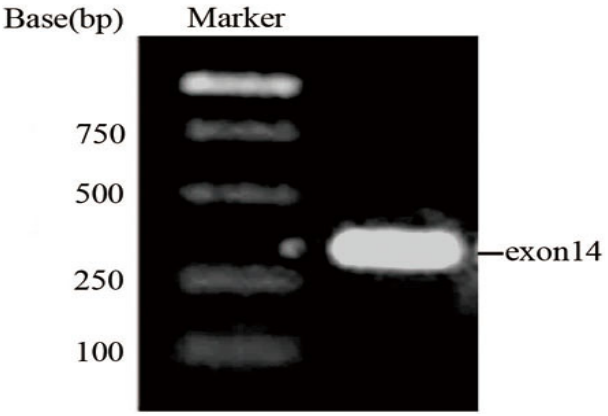

Figure 2. Electrophoresis Results of hpot1 Exon14 PCR Products in HeLa Cell Line

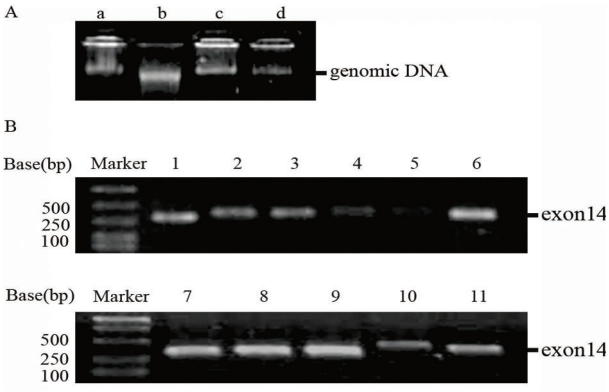

Figure 3. The PCR Results of Hpot1 Exon14 in Different Cancer Tissues. (A) the cancer tissues genomic DNA extract. a: uterine cervix cancer, b: endometrial cancer, c: carcinoma of bladder, d: esophageal carcinoma. (B) representative agarose electrophoresis images of the hpot1 exon14 PCR in all kinds of cancer tissues. 1-9: all kinds of cancer tissues. especially, 5: esophageal carcinoma, 9: endometrial cancer

that the total molecular weight will become bigger. And the electrophoresis speed of the binding complex would change much slower and formed visible hysteresis band (Figure 1 A. lane 2). Certainly, the excess probes formed the free fore-lying band.

Then, we extracted total nuclear proteins of HeLa cell and performed EMSA to detect expression of hPOT1. The results showed that hPOT1 was expressed in HeLa cell line. The signal strength of hysteresis band was gradually enhanced as the amount of nuclear protein increased (Figure 1 B). The free probes emerged anterior hPOT1 band.

hpot1 has 22 exons and 5 introns, we selected exon14 as the representative of *hpot1*. The exon14 (ENSE00002489750) located in chromsome7 from 124,481,232 to 124,481,027 and its full length (ENSE00002489750) is 206 bases. As the exon14 sequences desigened the primers and employed normal PCR to detect the *hpot1* expression in HeLa cell line. The results showed that the PCR products have a bright lane between 250bp and 500bp, it was consistent with the theory expectation (276bp, Figure 2).

After extracting total genomic DNA of different cancer tissues. 2μL samples were taken in 0.8% agarose gel electrophoresis and ran for 4 h. The results showed that a single high molecular weight band existed near the

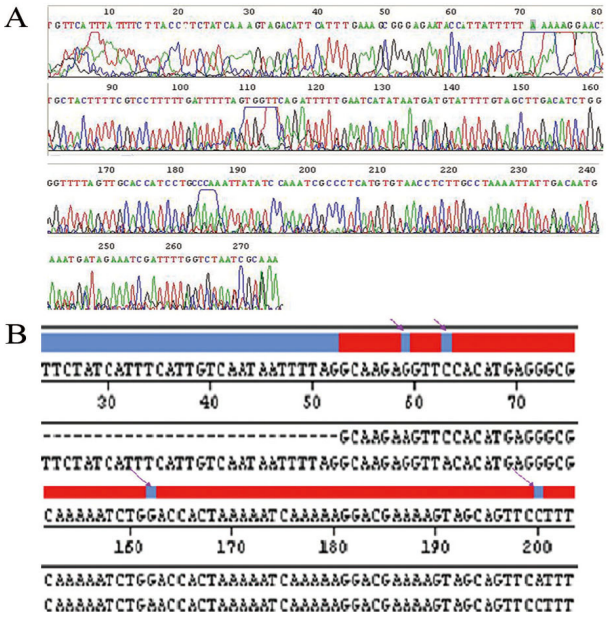

Figure 4. Sequencing Atlas of Mutant Type Exon 14 of Hpot1 in Endometrial Cancer. (A) the sequencing atlas of mutant type exon 14 of hpot1 in one endometrial cancer. (B) Mutant analysis on the mutant type of hpot1 exon 14 in endometrial cancer compared with the wild type pot1 exon14 with DNAstar 5.0 software

Table 1. Exon14 of Hpot1 Point Variations in Different Tumor Samples

Tumors samples	Sample number	Point variations number/total number)	Point variations
uterine cervix cancer	3	0/3 (0)	N
ovarian cancer	3	0/3 (0)	N
carcinoma of bladder	3	0/3 (0)	N
renal carcinoma	3	0/3 (0)	N
breast carcinoma	1	0/1 (0)	N
endometrial cancer	3	2/3 (66.7%)	6(A→G), 10(C→A), 107(G→A), 146(A→C)
carcinoma of gingiva	1	0/1 (0)	N
lung cancer	1	0/1 (0)	N
rectal cancer	1	0/1 (0)	N
esophageal carcinoma	1	0/1 (0)	N
Total	20	3/20	

kind of hole (Figure 3 A). It revealed that the genomic DNA were integrity extracted through traditional Phenol-chloroform method. According to *hpot1* exon14 PCR amplified systems, we detected different cancer tissues, including uterine cervix cancer, ovarian cancer, carcinoma of bladder, renal carcinoma, breast carcinoma, endometrial cancer, carcinoma of gingival, lung cancer, rectal cancer, esophageal carcinoma tissues. Results showed that the PCR products have bright lanes between 250bp and 500bp, it was consistent with the anticipation size (Figure 3B). The highest expression of *hpot1* exon14 was endometrial cancer (Figure 3B lane 9), in contrast the lowest was esophageal carcinoma (Figure 3B lane5).

PCR products were enriched and purified by Promega Wizard SV Gel and PCR Clean-Up kit. Then the enriched products were sent to Corp to identity the DNA sequence. Employed DNAstar 5.0 to analysis the base sequence (Figure 4 A). The results revealed that most cancer tissues exon14 do not exist point mutation. In contrast, exon14 sequence present gene point mutation in 2 out of 3 cases endometrial cancer (66.7%) (Table 1). The point mutations separately were 6 (A→G), 10 (C→A), 107 (G→A), 146 (A→C) (Figure 4 B). It gave a clue that those mutations may have some function in endometrial cancer.

Discussion

hPOT1 is a specialized G-strand binding protein, which binds to telomeric ssDNA directly via its OB folds (BaumannCech, 2001; Lei et al., 2004; Loayza et al., 2004). hPOT1 protects TRF2 -induced loss of telomeric single -stranded overhangs and chromosomal instability (Yang et al., 2005). For hPOT1 normal function was correlated with its expression, this study we employed EMSA to detect the expression of hPOT1 in HeLa cell line. EMSA is a radiol technique which domain analysis the DNA binding proteins or RNA binding proteins (Smith et al., 2009). It is based on the observations that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid. So EMSA is an important technique underlying a wide range of qualitative and semi-quantitative analyses for the characterization of interacting systems (Hellman et al., 2007). EMSA have been applied in detecting telomere proteins contains hPOT1 (BaumannCech, 2001; Kelleher et al., 2005). Because hPOT1 binds to telomeric ssDNA TTAGGG repeats, so we designed oligo probes of (TTAGGG)4 (total 20 bp). Here we have revealed that hPOT1 is strongly expressed in HeLa cell line. Although there are some reports on the expression of hPOT1 in cancer cell line, little message about the expression of hPOT1 in HeLa cell line. hPOT1 express in gastric cell lines SGC-7901 and MKN28, which may involved in invasion, proliferation and apoptosis (Fujii et al., 2008; Wan et al., 2011). In addition, hPOT1 is expressed in larynx squamous carcinoma cell lines Hep-2 and Hep-2R (Tang et al., 2009). We performed EMSA to analysis the hPOT1 expression and found that hPOT1 is expressed in HeLa cell line. It gave a clue that hPOT1 may be correlated with cervical cancer.

Subsequently, we employed normal PCR to analysis the expression of *hpot1* in HeLa cell line. For *hpot1* have 22 exons (Baumann et al., 2002), any encoding exon can represent the whole expression of *hpot1*. For high frequency alternative splicing exons, exon 8 to exon 17 are very important. We selected exon 14 as the PCR target fragment. The results showed that a strong PCR bands appeared between 250bp and 500bp, which was consistent with the exon14 sequence length. So we could think that the whole *hpot1* gene was exist in HeLa cell line. The results was consistent with the aforementioned EMSA results.

So far, many studies have demonstrated hPOT1 is overexpressed in a lot of human cancers, including non-small cell lung cancer (Lin et al., 2006), gastric cancer (Fujii et al., 2008; Wan et al., 2011), breast cancer (Savage et al., 2007). Those researches revealed that hPOT1 has close relationship with the occurrence or development of cancer, which our finding was a complementary to those researches. In addition, many reports have demonstrated that genetic variations emerge in several cancer. Savage, S. A. have reported that there are 4 single nucleotide polymorphisms(SNP) of *hpot1* gene in breast cancer in

spite of no tumor genesis risk (Savage et al., 2007). Varadi, V et al independently found similarity results in breast cancer (Shen et al., 2010). Jin Eun Choi et al found that 8 SNPs of *hpot1* in which 2 SNPs are the risk factors in lung cancer (Choi et al., 2009). Hosgood, H. D also found *hpot1* genetic variations in lung cancer tissues (Hosgood et al., 2009). Nan, H et al have demonstrated that there are *hpot1*genetic point variations in skin cancer (Nan et al., 2011). We firstly detected *hpot1* gene exon14 in various of cancer, including uterine cervix cancer, ovarian cancer, carcinoma of bladder, renal carcinoma and so on. Then we amplified the *hpot1* exon14 and identity the sequence. Finally sequence analysis was performed by DNAStar 5.0 software. The results showed that most cancer tissues exon14 do not exist point mutation. In contrast, exon14 sequence present gene point mutations in 2/3 cases endometrial cancer. The point mutation separately were 6 (A→G), 10 (C→A), 107 (G→A), 146 (A→C). The exons size and the position of splice sites are highly conserved for *hpot1* gene is a house keeping gene. So our findings gave a clue that those genetic mutations may have some unknown function in endometrial cancer progression. Whether those variations are SNPs or not require our further investigation.

In conclusion, our results suggest that hPOT1 is expressed in HeLa cell line. Furthermore, four gene point variants were found in endometrial cancer, the probability of gene point variants was obviously higher in endometrial cancer than other cancers. hPOT1 genetic abnormality may be involved in the genesis and development of gynecological cancers, especially in cervix and endometrial cancers.

Acknowledgements

The authors declare that they have no competing interests.

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