

Original Article

Mutations and reduced expression of p53 gene are involved in HPV-independent oncogenesis of cervical cancer

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Abstract: Cervical cancer is one of the most prevalent malignancies in women, which has human papillomavirus infection as the major risk factor. Here we report the establishment of a new HPV-negative cervical cancer cell line and characterization of genetic alterations relevant to the pathogenesis of cervical cancer. A new cervical cancer cell line was established by extensive culture and natural selection of cells a cervical cancer sample of a 53-year woman. Human papillomavirus was detected by ELISA and PCR. The expression levels of p53 and k-ras were analyzed by immunocytochemical staining, the mutations of p53 and k-ras were assessed by PCR-sequencing, the sensitivity to cisplatin and taxol was measured by MTT assay, chromosomal anomaly was detected by karyotyping. The newly established cervical cancer cell line (CTCC-1) did not have detectable HPV DNA or HPV viral particle. CTCC-1 cells had massive chromosomal changes and harbored heterozygous mutations at 870 C>T (protein Pro223Leu) and 1022 G>T (protein Val274Phe) of p53 gene (NM_000546) with reduced p53 expression compared to C33A cells whereas k-ras was not mutated nor inhibited in CTCC-1 cells. CTCC-1 cells were more sensitive to cisplatin and taxol than Hela but more resistant to taxol than C33A cells. Overexpressing wild type p53 significantly increased the sensitivity of CTCC-1 to those chemotherapy agents. Mutant p53 might be responsible for the oncogenesis of some HPV-negative cervical cancers.

Keywords: Cervical cancer, p53, HPV-negative, chemo-resistance, nucleotide salvage

Introduction

Cervical cancer is one of the most prevalent malignancies among women with significantly new cases and resultant deaths each year in developing countries [1]. Persistent infection of high-risk subtypes of human papillomavirus (HPV) is the underlying factor for the pathological progression of cervical cancer, which accounts for about 90% of cervical cancer cases [1, 2]. Among HPV proteins, oncogenic E6 protein physically interacts with and promotes the degradation of p53 proteins and other pro-apoptotic proteins [3, 4] whereas E7 deactivates retinoblastoma proteins (pRB) [4, 5], disrupting the normal cell cycle control and allowing neoplastic growth. Even though at fairly low prevalence, evidences have shown the existence of HPV negative cervical cancers [6-8]. Due to its infrequent occurrence, the pathogenesis of HPV-negative cervical cancers

has not attracted much attention academically or clinically. Here we report the establishment and characterization of a new HPV-negative cervical cancer cell line and demonstrate that p53 mutations may play a critical role in HPV-independent cervical cancers.

Materials and methods

Isolation of cervical cancer cells

The cancer tissue was obtained from a 53 year old Han Chinese woman (Nanchang, China) undergoing cervical cancer resection surgery. The patient had highly differentiated keratinizing squamous cell cancer of cervix with locally 6 mm deep invasion. The study protocol was reviewed and approved by institutional review board of Nanchang University. Informed consent was obtained from the patient. Cancer tissue was minced and digested with 0.025%

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trypsin-EDTA overnight at 4°C. The following day, it was digested at 37°C for 45 min and dispersed with pipetting few times. After adding 5 ml EMDM with 10% FBS, the sample was passed through 120 mesh sieve and then centrifuged at 250 g for 5 min to remove remained tissue pieces. The cells were seeded at a density of 1.5 million per 10 cm plate. After 3 passages, a portion of the cells were seeded at a much diluted density (50000 per 10 cm plate) to isolate naturally immortalized clones. One such clone was named CTCC-1 and subsequently characterized.

Establishment of wild type p53 stably transfected CTCC-1 cells

CTCC-1 cells were transfected with pQCXIP empty vector or pQCXIP-p53 and then selected with 3 µg/ml puromycin (ThermoFisher, Shanghai, China). Single clones were selected and further treated with puromycin until no dead cells appeared between passages.

Cell culture

CTCC-1 cells were cultured in RPMI-1640 medium and C33A and Hela cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO, Shanghai, China), 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Beyotime, Shanghai, China) at 37°C in humidified air with 5% CO₂. The cells were split when necessary.

Detection of HPV

The presence of HPV in cervical cancer cells was detected with a commercial human papillomavirus ELISA Kit (E0787h, Eiaab Science, Wuhan, China) according to manufacturer's protocols. The HPV DNA was detected by a previously described PCR procedure with GP5+/GP6+ primer set [9].

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, and blocked in 10% normal goat serum for 1 hr at room temperature. The cells were then incubated with anti-p53 (ab28, Abcam, Cambridge, MA) or anti-ras (ab55391, Abcam) at 4°C overnight

and then DyLight 680 conjugate goat anti-mouse IgG secondary antibody (35518, Pierce Antibody) away from light for 1 hr at room temperature before counterstained with DAPI and mounted.

Karyotyping

The density of CTCC-1 cells was adjusted to 10⁵/ml and seeded in five 10 cm dishes. Colcemid (Sigma, St Louis, MA) (a final concentration of 0.2 µg/ml) was added and shaking at 37°C for one hour before the cells were digested and collected into 10 ml centrifuge tubes, which were centrifuged 1000 rpm for 10 minutes. The supernatant was removed, and cells were resuspended in 8 ml prewarmed 75 mmol/L KCl and incubated in 37°C water bath for 40 min. The cells were then fixed in 2 ml freshly prepared fixative (3:1 (v/v) of methanol and acetic acid) at room temperature for 10 min and washed with 10 ml fixative three times before were placed on slides and dried 6 h at 60°C. The dried slides were immersed in trypsin (0.25%) about 1 min and then stained in Giemsa (Sigma) solution for 4-5 minutes before washed under running water and observed under a microscope. Karyogram analyses were performed using Leica CW4000 Karyo V1.1 software (Leica Imaging Systems, Cambridge, UK).

Drug resistance (MTT) assay

Cells (5000/well) were seeded in 96 well plates overnight before treated with 25 µM cisplatin or 1 nM taxol for 24 hr or otherwise specified time. Then 10 µl of 12 mM MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (ThermoFisher, Shanghai, China) to each well and incubated at 37°C for 3.5 hr. The culture medium was replaced with 100 µL of DMSO and thoroughly mixed. The plates were incubated at room temperature with shaking for 30 min before read at 540 nM.

Total RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from cells using RNAiso plus (Takara, Dalian, China) according to the manufacturer's instructions. The total RNA was reverse-transcribed into cDNA using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA), PCR amplification was per-

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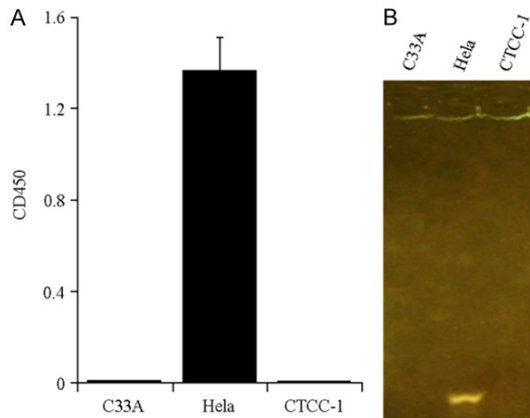


Figure 1. HPV was not involved in the pathogenesis of cervical cancer which CTCC-1 cell line was originated from. A. ELISA assay detected HPV virus in HeLa cells but not in C33A and CTCC-1 cells. B. HPV DNA was detected in HeLa cells but absent from C33A and CTCC-1 cells by specific PCR analysis.

formed using enzyme of Primer Star (Takara, Dalian, China) in a 50 μ l reaction containing 1 μ l of each primer, 0.5 μ l Primer Star, 10 ml buffer and 2 μ l of cDNA. The PCR primers were TGACGGAGGTTGTGAGG and GATAAGATGCTGAGGAGGG for P53, TTGACGCCTGTAATCCG and GAGCGAGACTCAGGTTTG for Kras, AAGAGGGCGTGGTGTATGTG and CAGGGGATTGGCTCCACTTT for ADA, CACTTAGGCTCGTTGCGGT and AGTGTCTTAGGCTTGTCTTCT for UCK1, TGCTTTCCTTGGTCAGGCAG and TTCGTGGGGTCCTTTTACC for HPRT1, CGCCTATACCAAGAGGCTCG and AAGTAGCAGAGCCGACACAC for TK1, and CGTCTTCCCCTCCATCGT and GCCTCGTCGCCACATAG for β -actin. PCR was carried out on a ABI 7500 Fast (Applied Biosystems, Foster City, CA) with following program: 95°C 2 min followed by 40 cycles of 95°C 10 sec, 56°C 5 sec and 72°C 15 sec. The relative gene expression level was calculated using $2^{-\Delta\Delta Ct}$ method with β -actin as the internal control.

Detection of mutations in p53 and kras

Exons 5 to 8 of p53 gene and exons 2 to 4 of kras gene were PCR amplified from CTCC-1 cell genomic DNA and gel purified. The PCR products were sequenced using the PCR primers by Sangon (Shanghai, China). Amplification was performed with 95°C for 15 min, 35 cycles of 94°C, 30 sec, 58°C, 30 sec and 72°C, 30 sec. The primers used were ATCTGTTCACTTGTGCCCTG and AACCAGCCCTGTCGTCTCTC for p53 exon 5; AGGGTCCCCAGGCCTCTGAT and CACCCTTAACCCCTCCTCCC for p53 exon 6; CCAA-

GGCGCACTGGCCTCATC and CAGAGGCTGGGGCACAGCAGG for p53 exon 7; TTCCTTACTGCTTCTTGCTT and TGCCTGCTTGCTTACCTCG for p53 exon 8; AAGGTACTGGTGGAGTAT and AATGGTCTGCACCAGTA for kras exon 2; GAAGTAAAAGGTGCACTGT and TACTCCTTAATGTCAGCTTA for kras exon 3; GTTGTGGACAGGTTTTGA and TGCCCTCTCAAGAGACA for kras exon 4.

Construction of wild type p53 expression vector

The coding sequence of p53 gene was PCR amplified from pET15b-p53 (Plasmid 24859, Addgene, Cambridge, MA) with primers GGCA-CCGGTATGGAGGAGCCGCAGTCA and GCCGGA-TCCTCAGTCTGAGTCAGG. The PCR product was digested with AgeI and BamHI and inserted into pQCXIP (Clontech, Mountain View, CA) cut with the same enzymes. The inserted sequence was verified by DNA sequencing (Sangon, Shanghai, China).

Statistical analysis

Data were expressed as mean \pm standard error. The differences between CTCC-1 and other cell lines or treatment and control were analyzed by One-way ANOVA followed by pos-hoc Bonferroni assay or Student's t-test using Graphpad Prism 6. *P*-values less than 0.05 were considered as statistically significant.

Results

CTCC-1 is a HPV-negative cervical cancer cell line

CTCC-1 cells were established from cervical cancer tissue of a 53-year old Han Chinese woman by spontaneous immortalization. ELISA assay did not detect any HPV viral particle (**Figure 1A**). PCR with GP5+/GP6+ primer set did not detect HPV DNA either (**Figure 1B**).

CTCC-1 cells has massive genetic changes

Many chromosomal changes were detected in CTCC-1 cells. Arm loss was found in chromosomes 1, 18, and 22. Trisomy 4, 5, and 8, and tetrasomy 9 were observed (**Figure 2**).

CTCC-1 cells harbor heterozygous mutations in p53 gene with altered p53 protein level

The Exons 5-8 of p53 gene were PCR amplified and sequenced. Two heterozygous mutations were identified at the nucleotide 870 (T>C)

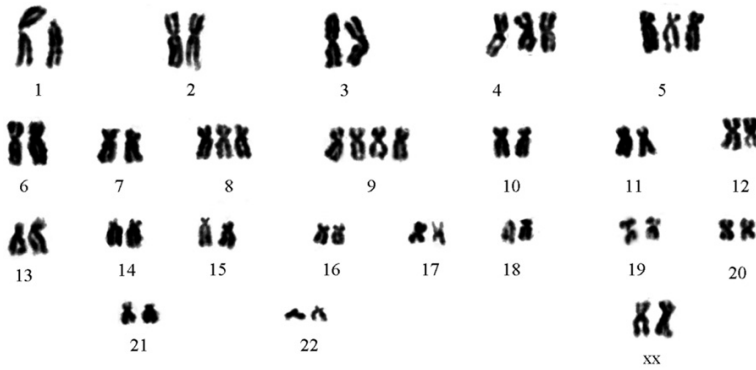


Figure 2. CTCC-1 cervical cancer cells underwent massive genetic changes. Karyotyping analysis detected arm loss in chromosomes 1, 18, and 22. Trisomy 4, 5, and 8, and tetrasomy 9 were also observed.

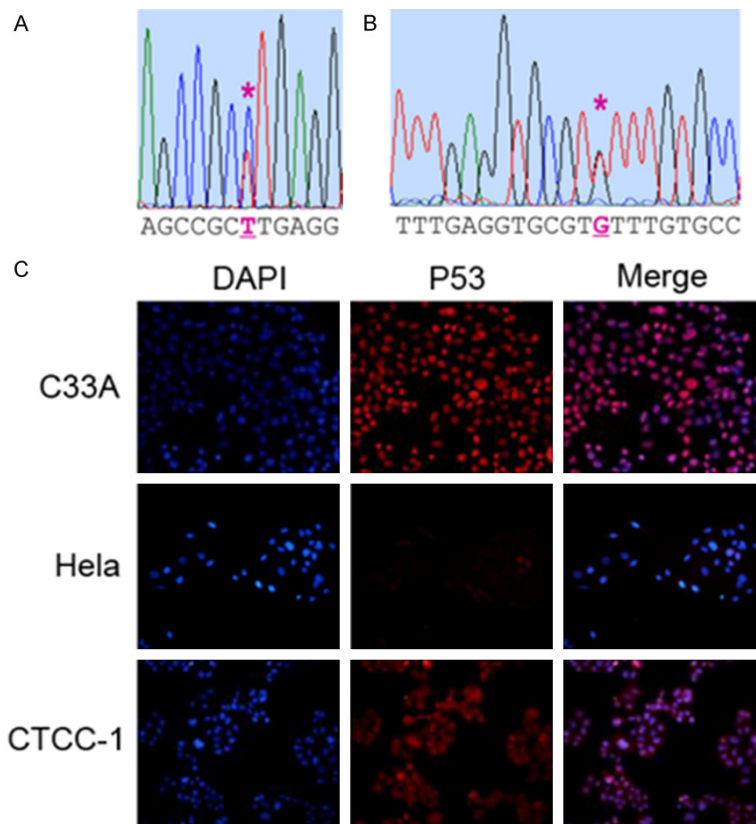


Figure 3. P53 gene of CTCC-1 cells was mutated with reduced expression. Two mutations were identified in p53 gene. Heterozygous mutations at nucleotides 870 (T>C). A. And 1022 (G>T). B. Were identified by PCR-sequencing, which resulted in Pro223Leu and Val274Phe mutant p53 protein. C. Immunofluorescence with anti-p53 antibody showed that p53 protein level of CTCC-1 cells was about 50% of that of C33A cells but significantly higher than Hela cells.

(**Figure 3A**) and 1022 (G>T) (**Figure 3B**) of p53 gene (NM_000546), which resulted in proline 223 and valine 274 of P53 protein changing into leucine and phenylalanine respectively.

Meanwhile, the p53 protein level of CTCC-1 cells was significantly lower than that of C33-A cells but much higher than that of Hela cells (**Figure 3C**). No mutation was identified in K-ras gene and its expression was similar to that of C33A and Hela cells (data not shown).

The expression of nucleotide salvage pathway genes was different in CTCC-1 cells compared to other cervical cancer cell lines

The genes involved in nucleotide salvage pathways were differentially expressed in CTCC-1 compared to other cervical cancer cell lines. ADA, TK1, and UCK1 mRNA levels of CTCC-1 cells were significantly lower than those of C33A cells whereas HPRT was substantially upregulated in CTCC-1 cells compared to C33A cells (**Figure 4**). On the other hand, the expression levels of ADA, HPRT, TK1, and UCK1 were markedly lower than those of Hela cells (**Figure 4**).

Wild type p53 increased the sensitivity of CTCC-1 to chemotherapy agents

CTCC-1 cells had similar resistance to cisplatin as C33A cells but significantly more sensitive to cisplatin than Hela cells (**Figure 5A**). On the other hand, CTCC-1 cells were markedly more resistant than C33A but substantially more sensitive than Hela cells to taxol (**Figure 5B**).

Next, whether wild type p53 could inhibit the chemoresistance of CTCC-1 was investigated. Ectopically expressed wild type p53 reduced the survival rate of CTCC-1 cells by 53% and

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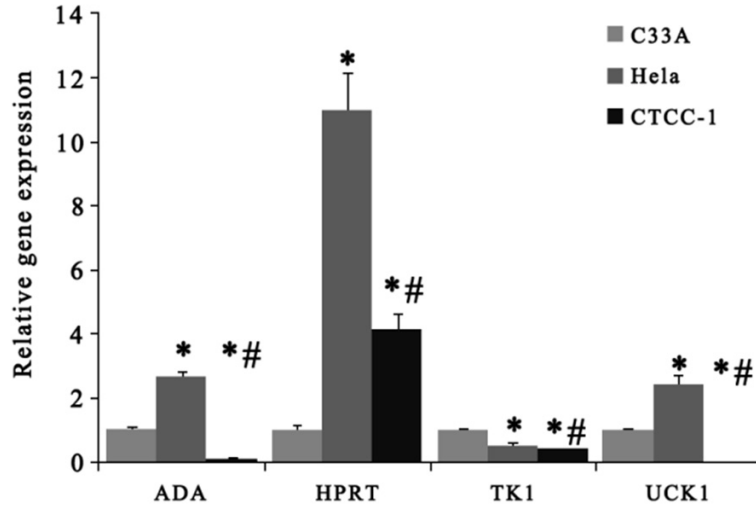


Figure 4. The expression pattern of nucleotide salvage genes was different from other cervical cancers. The mRNA levels of ADA, HPRT, TK1, and UCK1 were assessed by quantitative PCR. Data were expressed as mean \pm standard error from 3 independent experiments. *, $P < 0.05$ compared to C33A; #, $P < 0.05$ compared to HeLa.

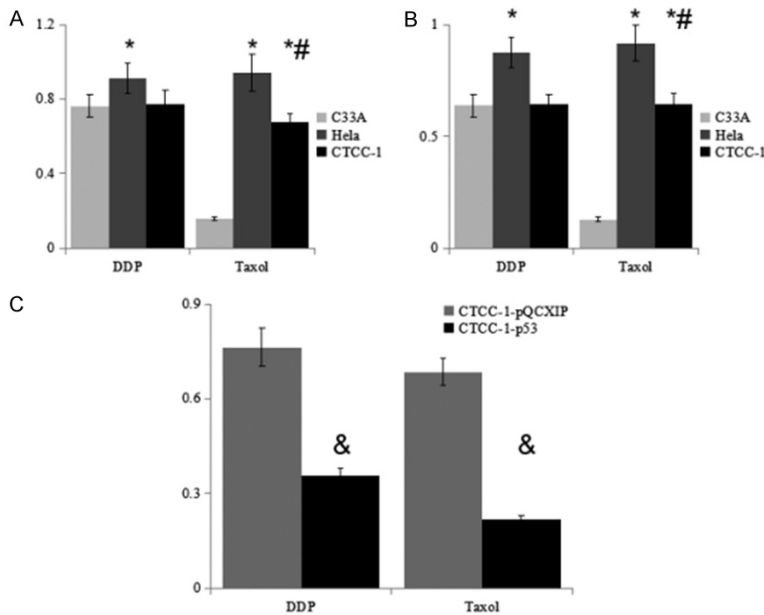


Figure 5. Wild type p53 increased the sensitivity of CTCC-1 cells to cisplatin and taxol. CTCC-1, C33A, and HeLa cells were treated with 25 μ M cisplatin or 1 nM taxol for 24 hr (A) or 48 hr (B) before analyzed with MTT assays. (C) MTT assay showed that CTCC-1 cells transfected with wild type p53 had significantly lower survival rate than CTCC-1 transfected with empty vector when treated with either 25 μ M cisplatin or 1 nM taxol for 24 hr. Data were expressed as mean \pm standard error from 3 independent experiments. *, $P < 0.05$ compared to C33A; #, $P < 0.05$ compared to HeLa. &, $P < 0.05$ compared to CTCC-1-pQCXIP.

68% when treated with cisplatin and taxol respectively (**Figure 5C**).

Discussion

Here we described a newly established human cervical cancer cell line, CTCC-1. This cell line was HPV-negative with changes on several chromosomes. K-ras was not mutated nor was its expression changed. However, p53 gene was mutated at nucleotide 870 (T>C) and 1022 (G>T) heterogeneously and its expression level was significantly lower than C33A cells. The expression pattern of genes involved in nucleotide salvage pathways in CTCC-1 cell was drastically different from other cervical cancer cell lines.

As persistent HPV infection has been considered as a necessary factor for developing cervical cancer [2] and accounted for around 90% of cases of grade 3 cervical intraepithelial neoplasia and invasive cancer [10], there rarely was new information about the pathogenesis of HPV-negative cervical cancers due to its scarcity. The current study isolated a cervical cancer cell line from a Chinese patient which was deemed HPV-negative based on following evidences. A specific ELISA assay did not detect any HPV viral particles in CTCC-1 cells compared to HeLa (HPV18) and C33A (HPV-negative) cells. Meantime, a sensitive PCR analysis [9] failed to detect HPV DNA in CTCC-1 cells. Third, the significant p53 protein level implied the lack of HPV in CTCC-1 cells as p53 protein levels were strongly depressed in HPV-positive cervical cancer cell lines [11].

The loss of proper function of p53 protein is the main event of the onset and development of

most cancers, which is caused by E6 oncoprotein-mediated p53 protein degradation in high risk HPV-caused cervical cancers [4, 23]. As the extremely high prevalence of HPV infection in cervical cancer patients, the HPV-independent oncogenic process of cervical tissue has been mostly ignored. This study reintroduced the issue of tumorigenesis of cervical tissues in the absence of high risk HPV and identified two heterogeneous mutations in p53 gene. These single nucleotide mutations and the reduction of p53 protein level might represent a series of major oncogenic events. The same p53 mutations have only been reported in prostate cancer cell line DU145 [12] and might be responsible for the inhibition of 4-hydroxynonenal induced apoptosis [13]. The Pro223Leu/Val274Phe p53 protein in DU145 cells conferred the resistance to docetaxel similar to p53-null of PC3 cells and introduction of wild type p53 into DU145 cells resulted in sensitivity to docetaxel and activation of intrinsic apoptosis pathway [14]. That the resistance of CTCC-1 to cisplatin and taxol could be inhibited by wild type p53 was in line with aforementioned observations and the current data demonstrated the possible mechanism that cervical cancer could developed in the absence of HPV infection.

Nucleotide salvage pathways have been shown to be involved in cancer growth and invasion [15, 16] and were regulated by both tumor suppressors (e. g. pRB and p53) and oncoproteins (e.g., c-myc) [16-19]. Gain-of-function mutant p53 proteins could promote the proliferation and invasion of cancer cells by circumventing normal metabolic processes [16, 20, 21]. Overexpressing p53Arg273His in Rat-1 cells caused significant reduction of hypoxanthine phosphoribosyl transferase (HPRT) and thymidine kinase (TK) activities whereas it increased the activities of adenosine deaminase and uridine kinase [17]. Mutant p53 protein regulated many nucleotide metabolism genes at transcriptional level [16], which was in support of our current findings that nucleotide salvage pathway genes were differentially expressed in cervical cancer cell lines with different p53 mutations and/or protein levels.

In conclusion, we established and characterized a new cervical cancer cell line, CTCC-1, which was originated from a Chinese patient. Massive genetic changes were detected on

chromosomal level and two particular single nucleotide mutations were identified in tumor suppressor p53 gene that resulted in Pro223Leu/Val274Phe mutant p53 protein. Meanwhile, p53 protein level of CTCC-1 was prominent but significantly less than that of C33A cells. The mutational and expressional changes of p53 might be responsible for the changes of nucleotide salvage pathway gene expression and resistance to chemotherapy agents. The other changes of CTCC-1 cells related or unrelated to p53 mutations are currently under systematic investigation.

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Disclosure of conflict of interest

None.

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