Significance of Mutation in the D-loop Region in Cervical Cancer

Y Zhao, J Wang, X Chen, Q Wu

ABSTRACT

Aim: *To investigate the mutation in the D-loop region of mitochondrial DNA in cervical cancer and its influence on the changes of reactive oxygen species (ROS) and cell cycle.*
Methods: *The D-loop region was amplified by PCR and sequenced. Reactive oxygen species and cell cycle were detected by flow cytometry in 40 specimens from cervical cancer and adjacent normal tissues. According to the sequence results, gastric cancer tissue was divided into mutation group and control group. Reactive oxygen species, apoptosis and proliferation in the two groups were compared.*
Results: *Among the 40 cervical cancer specimens, 21 mutations were identified in 12 patients, the mutation rate being 30%. There were four microsatellite instabilities in the mutations. No mutation was found in the adjacent tissues. Reactive oxygen species, apoptosis and proliferation in the mutation group were all significantly higher than those in the control group.*
Conclusion: *Mutation in the D-loop region plays a role in the genesis and development of cervical cancer.*

Key words: Cervical cancer, D-loop, DNA, mitochondria, reactive oxygen species

Importancia de la Mutación en la Región D-loop en el Cáncer Cervical

Y Zhao, J Wang, X Chen, Q Wu

RESUMEN

Objetivo: *Investigar la mutación en la región D-loop del ADN mitocondrial en el cáncer cervical y su influencia en los cambios de las especies reactivas de oxígeno (ROS) y el ciclo celular.*
Métodos: *La región D-loop fue amplificada mediante PCR y secuenciada. Las especies reactivas de oxígeno y el ciclo celular fueron descubiertos mediante citometría de flujo en 40 espécimenes de cáncer cervical y los tejidos normales adyacentes. Según los resultados de la secuencia, el tejido canceroso gástrico fue dividido en un grupo de mutación y un grupo control. Se compararon las especies reactivas de oxígeno reactivo, la apoptosis y la proliferación en los dos grupos.*
Resultados: *Entre los 40 especimenes de cáncer cervical, se identificaron 21 mutaciones en 12 pacientes, para una tasa de mutación del 30%. En las mutaciones se presentaron cuatro inestabilidades de microsatélite. No se encontró mutación alguna en los tejidos adyacentes. Las especies reactivas de oxígeno, la apoptosis y la proliferación en el grupo de mutación fueron todas significativamente mayores a las del grupo de control.*
Conclusión: *La mutación en la región D-loop desempeña un papel en la génesis y desarrollo del cáncer cervical.*

Palabras claves: Cáncer cervical, D-loop, ADN, mitocondria, especies reactivas de oxígeno

West Indian Med J 2010; 59 (3): 291

INTRODUCTION

It has been proven that multiple factors and gene mutations take part in the occurrence and progression of cancer. Although great achievement has been made in oncogenes and cancer suppressor genes, many questions cannot be explained by alteration of nuclear genes. In recent years, mutation of mitochondrial DNA (mtDNA) has been associated with the

From: Department of Gynaecologic Oncology, Jiangsu Cancer Hospital and Research Institute, Nanjing 210009, Jiangsu Cancer Hospital and Research Institute, Nanjing 210009, Jiangsu Province, China.

Correspondence: Drs Y Zhao\Q Wu, Department of Gynecologic Oncology, Jiangsu Cancer Hospital and Research Institute, Nanjing 210009, Jiangsu Province, China. E-mail: njzyb72@sina.com.

occurrence and progression of tumour and is regarded as a possible causitive factor for cancer (1).

Human mtDNA is a closed double strand circular molecule of 16569bp, coding 13 proteins, 22 tRNAs and 2 rRNAs. Its D-loop is a non-coding region, containing some important sequences such as the promoter for heavy chain replication. Because of the properties of its structure as well as its mechanism of DNA replication and injury repair, the mutation frequency of mitochondria is 10 to 100 times higher than that of nuclear DNA. In recent years, the instability, gene mutation or abnormal expression of mitochondrial genome has been detected in many kinds of malignant cancer tissues (2, 3). The mitochondrial D-loop is a hotspot for gene mutation in cell lines of colonic and rectal cancer. But there is a difference in mutation frequency of the D-loop among different tumours (4–7).

We studied the region of mtDNA D-loop which had been amplified by PCR and then sequenced with samples from cancer tissues and corresponding normal tissues of 40 cervical cancer patients. Reactive oxygen species (ROS) and the cell cycle stage were detected with flow cytometry. The samples were divided into a mutation group and a control group according to the mutations. We compared the level of ROS and the cell cycle in two groups to evaluate the influence of mutations of D-loop on ROS and cell cycle. The purpose was to investigate the influence of mutation of the D-loop on cell carcinogenesis and progression of cervical cancer.

SUBJECTS AND METHODS

Forty surgical samples of cervical cancer were selected from hospitalized patients in the Department of Gynaecologic Oncology of Jiangsu Cancer Hospital from June to November, 2007. All patients, including those with squamous carcinoma [33] and adenocarcinoma [7], were diagnosed by biopsy. Neither radiotherapy nor chemotherapy was performed before operation. Their ages varied from 21 to 56 years and averaged 43.2 years.

A region without bleeding was carefully selected. A block of 1.0 cm^3 fresh cancer tissue was cut and stored in a -70°C refrigerator. Other tissues were cleaned with phosphate buffer solution (PBS). Cell suspension was prepared by mechanical grinding and filtered through a net and cancer cells were separated by centrifugation (proved by Rye's dyeing). A block of 1.0 cm^3 normal cervical tissue was cut and cell suspension was prepared according to the same procedure.

Half of 1 ml cell suspension was put into a test tube, centrifuged for 5 minutes at 1500 r/minute, washed three times with normal saline, water and cell debris were removed by centrifugation for 3 minutes at 500–800 r/minute. Cells were fixed with 2–4ml 1% polyformaldehyde and centrifuged for 10 minute at 1500 r/minute. The supernatant was discarded and the pallet was resuspended in 2–4 ml 0.1% Triton-X-100 for three minutes and centrifuged. The supernatant was discarded and resuspended in 1–2 ml 0.01% RNase and vortexed for 30 minute in 37°C water bath. One ml 0.05% PI solution was added to dye the DNA for 30 minutes. The cell cycle of cancer cells and normal gastric cells was measured by FACS (Vantage SE, BD, America). The fluorescence signal was processed by multicycle analytical software for cell cycle.

DCFH-DA (from Sigma Company) was dissolved in 95% ethanol to a concentration of 5 mmol/L and stored at 4°C in the dark and diluted to 5 μmol/L with PBS before use (8). Two hundred μl cell suspension (1×10^6/ml) was put into a test tube, washed twice with PBS and centrifuged for 5 minutes at 1500 r/minute. The supernatant was discarded and the pallet was resuspended in 2 ml 5 μmol/L DCFH-DA (2 ml PBS for the contrast groups), vortexed for 20 minutes in 37°C water bath and centrifuged for five minutes at 1500 r/minute. The supernatant was discarded and the cells were resus-pended in 600 μl PBS. The intensity of DCF green fluores-cence was measured after DCFH-DA reaction with FACS. The wavelength of stimulation sub-laser within the FACS was 488 nm and the power was 10mW. The results were expressed as mean fluorescence intensity (MFI). DNA ex-traction was carried out according to the protocol of the reagent kit (Promega, America).

The sequences of primers are listed in Table 1. The total volume of PCR reaction was 50 μl, including 1μl of each primer (20 pmol/μl), 5 μl of 10×PCR buffer, 5 μl of dNTPs (2 nmol/L), 0.4 μl of ExTaq DNA polymerase (5 U/μl) and 100 ng of extracted DNA sample. PCR reaction was carried out using PCR instrument (Perkin Elmer 2400, America). The initial denaturation was at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45s, a renaturation at 56°C for 45s, and extension at 72°C for 90s. The final extension was at 72°C for 7 minute.

Table 1: Primers for amplifying and their nucleotide sequences

Primers	nucleotide sequence
Upper nt15791-15810	5′-ATCATTGGACAAGTAGCATC-3′
Down nt725-706	5′-GGTGAACTCACTGGAACGGG-3′

Two μl of PCR product was loaded on 1.5% agrose gel for electrophoresis. If the mtDNA D-loop region was successfully amplified, a picture was taken (Fig. 1) and the PCR product was purified with an instant PCR product purification kit (Promega, America) and quantified with a spectrophotometer.

The sequencing reaction was completed with the sequencing kit of end termination by fluorescence labelled ddNTPs (ABI, America). The total volume of sequencing PCR reaction was 10 μl, including 1 μl of sequencing primer, 6 μl of kit mixture, 3 μl of purified PCR product. Three sequencing primers (Table 2) were used to divide the replicated D-loop region into three overlapped segments. The reaction condition was as follows: the initial denaturation step was at

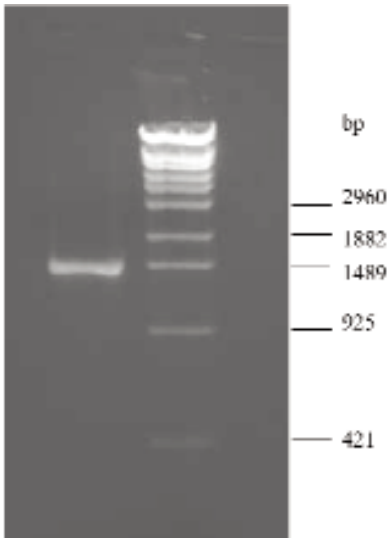

Figure 1: PCR amplification of D-loop region in mtDNA
Lane1: PCR product of D-loop region in mtDNA; lane 2: marker

Table 2: Primers for sequencing and their nucleotide sequences

Primers	nucleotide sequence
Upper nt111-nt130	5′-ACCCTATGTCGCAGTATCTG-3′
Upper nt16328-16347	5′-CGTACATAGCACATTACAGT-3′
Down nt16540-16514	5′-GTGGGCTATTTAGGCTTTATGACCCTG-3′

96°C for one minute, followed by 40 cycles at 96°C for 10s and at 55°C for two minutes. The sequencing reaction product was precipitated by 70°C ethanol and loaded on a ABI Prism 310 sequencer (Perkin Elmor, America)

Taking the sequence of mtDNA D-loop from Cambridge sequence (9) as criterion, a comparison was made betweeen the sequences of cancerous tissue and those of normal tissue. If the mtDNA D-loop sequence from cancerous tissue was different from normal tissue, the alteration was regarded as gene mutation.

Other data were expressed as mean ± SD. Comparison between groups was carried out by *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Twenty-one gene mutations were found in the cancerous tissue from 12 patients, among which four were microsatellite instabilities. Thus the mutation rate of mtDNA D-loop in the specimens of cervical cancer was 30% (Table 3).

Table 3: Mutations in D-loop region of mitochondrial DNA in gastric cancer

Location	Cambridge sequence	Cancer nucleotide Tissue nucleotide	Normal
16084	G	A	G
16358	C	T	C
16359	T	C	T
16366	T	C	T
16401	C	A	C
16438	G	A	G
16465	A	G	A
16492	A	deletion	A
16552	A	G	A
41	C	T	C
63	T	C	T
72	T	C	T
167	C	G	C
255	G	A	G
277	C	G	C
303	$(C)_7$	$(C)_8$	$(C)_7$
303	$(C)_7$	$(C)_9$	$(C)_8$
392	T	C	T
491	C	G	C
514	$(CA)_5$	$(CA)_6$	$(CA)_5$
567	$(C)_6$	$(C)_7$	$(C)_6$

Cell cycle and apoptosis could be detected by flow cytometry synchronously. In cell cycle, DNA was synthesized in synthesis(S) phase. As a result, the percentage of cells in synthesis phase could reflect cell proliferation. As shown in Table 4, level of ROS, rate of cell apoptosis and proliferation in the mutation group were higher than those in the controls ($p < 0.05$).

Table 4: ROS level and cell cycle in mutation group and control group (mean ± SD)

	ROS (MFI)	apoptosis (%)	G_0/G_1(%)	S (%)	G_2/M(%)
mutation group n = 7	178.2 ± 11.4[a]	13.1 ± 1.4[a]	55.2 ± 4.9	24.3 ± 2.1[a]	20.6 ± 2.1
control group n = 13	125.6 ± 10.8	7.5 ± 0.5	61.2 ± 4.7	21.2 ± 1.9	17.9 ± 1.3

[a] $p < 0.05$ *vs* control group

DISCUSSION

There was only 1120nt in the D-loop of mtDNA but 21 mutations were detected in the 40 cervical cancer patients, indicating that the D-loop of mtDNA is a fragment with a high mutation rate.

Among the 21 gene mutations found in this work, four were microsatellite instabilities. Wang (10) reported that 25.4% of cervical cancers, 48.4% of endometrial cancers, 21.9% of ovarian cancers and 29.4% of breast cancers carried one or more mitochondrial microsatellite instability (mtMSI)

which was frequently detected in the D-loop region but rarely occurred in the coding region. A relatively long C tract interrupted by a T residue is the mtMSI hot spot in all four types of cancer studied. MtMSI is correlated with nuclear MSI. Since nuclear MSI induces gene mutation in the coding region, mtDNA mutation plays its role in the process of cancer genesis and progression by cooperating with the alteration of some nuclear genes.

Although a non-coding region, the mtDNA D-loop contains the initial site of heavy chain replication and the promoters for heavy and light chain transcription. Thus D-loop is responsible for the regulation of mtDNA replication and transcription, its mutation leads to mutations in the coding region and change of protein synthesis, and finally affects the function of the respiration chain which hampers the energy supply of cells and produces a large amount of ROS. Reactive oxygen species results in injury to the genome and then induces cancer.

A high level of ROS is toxic through activating cell apoptosis and causing injuries to the genome. Reactive oxygen species might regulate cell apoptosis by the following ways: ROS is the message molecule of some transcription factors (such as Apaf-1) and can activate some useful components of cell apoptosis (11). The increase of ROS is often accompanied with the decrease of intracellular anti-oxidants, resulting in cell apoptosis (2). Most people believe that ROS is necessary for cell apoptosis. High levels of ROS inspires cell necrosis or drives cells from apoptosis to necrosis (12, 13).

Reactive oxygen species not only participate in the process of cell apoptosis but also is a kinetin for cell division that promotes nuclear DNA mutation, cell mitosis and selective growth of tumour cells. Reactive oxygen species is relatively stable and easy to diffuse within cells and exist universally in various cell types. The formation and elimination of them are under strong cellular regulation. All the above properties make ROS extraordinarily appropriate for second messengers (14–15). The level of intracellular ROS increases under extracellular stimulation signals such as cytokine and growth factor. Then they take part in cellular signal transduction. There exist two research hotspots at present for the relation between ROS and cell proliferation. One is the activation of the MAP kinase family to promote cell mitosis, the other is activation of transcription factors such as NF-κB to facilitate gene expression.

In conclusion, the mutation in the D-loop takes part in carcinogenesis and progression of cervical cancer through the effect of increased ROS.

REFERENCES

1. Copeland WC,Wachsman JT, Johnson FM, Penta JS. Mitochondrial DNA alterations in cancer. Cancer Invest 2002; **20:** 557–69.
2. Dasgupta S, Hoque MO, Upadhyay S, Sidransky D. Mitochondrial cytochrome B gene mutation promotes tumor growth in bladder cancer. Cancer Res 2008; **68:** 700–6.
3. Lee HC, Hsu LS, Yin PH, Lee LM, Chi CW. Heteroplasmic mutation of mitochondrial DNA D-loop and 4977-bp deletion in human cancer cells during mitochondrial DNA depletion. Mitochondrion 2007; **7:** 157–63.
4. Bragoszewski P, Kupryjanczyk J, Bartnik E, Rachinger A, Ostrowski. Limited clinical relevance of mitochondrial DNA mutation and gene expression analyses in ovarian cancer. JBMC Cancer 2008; **8:** 292.
5. Sharma H, Singh A, Sharma C, Jain SK, Singh N. Mutations in the mitochondrial DNA D-loop region are frequent in cervical cancer. Cancer Cell Int 2005; **5:** 34.
6. Fliss MS, Usadel H,Caballero OL,Wu L, Buta MR, Eleff SM et al. Facile detection of mitochondrial DNA mutations in tumor and body fluids. Science 2000; **287:** 2017–19.
7. Lewis PD, Fradley SR, Griffiths AP, Baxter PW, Parry JM. Mitochondrial DNA mutations in the parotid gland of cigarette smokers and non-smokers. Mutat Res 2002; **518:** 47–54.
8. Cai L, Gao S,Yang Y, Zheng D, Wang W, Zhang G et al. Study on the relationship between apoptosis and reactive oxygen species of cancer cell lines induced by anticarcinogens. Zhonghua Xueyexue Zazhi 2001; **22:** 249–51.
9. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J et al. Sequence and organization of the human mitochondrial genome. Nature 1981; **290:** 457–65.
10. Wang Y, Liu VW, Tsang PC, Chiu PM, Cheung AN, Khoo US et al. Microsatellite instability in mitochondrial genome of common female cancers. Int J Gynecol Cancer. 2006; **16 (Suppl 1):** 259–66.
11. Lovat PE, Ranalli M, Corazzari M, Raffaghello L, Pearson AD, Ponzoni M et al. Mechanisms of free-radical induction in relation to fenretinide-induced apoptosis of neuroblastoma. J Cell Biochem 2003; **89:** 698–708.
12. Jonassen JA, Cao LC, Honeyman T, Scheid CR. Mechanisms mediating oxalate-induced alterations in renal cell functions. Crit Rev Eukaryot Gene Expr 2003; **13:** 55–72.
13. Hsiao PN, Chang MC, Cheng WF, Chen CA, Lin HW, Hsieh CY et al. Morphine induces apoptosis of human endothelial cells through nitric oxide and reactive oxygen species pathways. Toxicology 2009; **256:** 83–91.
14. Zhang R, Humphreys I, Sahu RP, Shi Y, Srivastava SK. In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. Apoptosis 2008; **12:** 1465–78.
15. Madan E, Prasad S, Roy P, George J, Shukla Y. Regulation of apoptosis by resveratrol through JAK/STAT and mitochondria mediated pathway in human epidermoid carcinoma A431 cells.Biochem Biophys Res Commun 2008; **377:** 1232–7.