p53 Tumor Suppressor Gene Mutation in Ovarian Cancer in Thai Patients

NEELOBOL NEUNGTON, M.D.*,
CHAIRAT LEELAPHATANADIT, M.D.**,
RUNGTHIP SOIAMPORNKUL, M.Sc.*
SOMCHAYA NEUNGTON, M.D.**,
CHONGDEE DANGRAT, M.Sc.**,

Abstract

Objectives : To characterize molecular mutations of *p53* gene in Thai ovarian cancer and compare the mutations with their pathological and clinical findings.

Material and Method : Direct DNA sequencing of hot spot region of *p53* gene (exons 5 to 8) from 28 primary ovarian cancer tissues, 2 metastatic tumors and their paired blood samples was performed. The detected mutations were compared to the pathological and clinical findings and responsiveness to treatments after 36 months of follow-up.

Results : One insertion and 4 point mutations in exon 5 of *p53* gene were found in 5 out of 28 (18%) ovarian cancer patients. There was no mutation in the paired blood samples. The histological types of the detected tumors were 3 endometrioids and 2 serous cystadenocarcinomas. All 5 patients were in stage I to IV disease and showed overall 4 out of 5 (80%) complete response until 36 months after surgery followed by chemotherapy, compared to 14 out of 28 (50%) of complete response in all cases of ovarian cancer.

Conclusion : The authors found 5 cases of ovarian cancer patients with *p53* gene mutations giving the same response to complete standard treatment as all cases. Significant factors affecting responsiveness of these patients depended more on stages, grades and histological cell types of the cancer.

Key word : Ovarian Neoplasm, Gene *p53* Point Mutation, Molecular Sequence Data, Prognosis

NEUNGTON N, NEUNGTON S,
LEELAPHATANADIT C, DANGRAT C, SOIAMPORNKUL R
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* Department of Biochemistry,
** Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Ovarian cancer is the leading cause of death of all gynecologic malignancies[1,2]. In Thailand, it ranks fifth for the incidence of all female cancers[3]. In the USA it is the fourth most frequent cause of cancer death in women. It has been estimated that, in the USA, one woman in 70 will develop ovarian cancer in her lifetime and one woman in 100 will die of this disease[1]. This is mainly because the tumor is often far advanced by the time of clinical diagnosis and also the nature of most cell types are highly malignant[1,4]. The overall 5 year survival rates of ovarian cancer vary dramatically by stages, from 42-70 per cent for early stage and to 4-13 per cent for advanced stages[4]. Within each stage, differences in survival rates are also due to the histological cell types and ages of the patients, with better survival in younger than in older patients with the same tumor type[1,4,5]. Approximately two-thirds of the patients are diagnosed at late stages since early stage disease is usually asymptomatic, and early detection by physical examination, and other conventional investigations such as blood chemistry or ultrasonography have not been completely helpful. The disease may occur at any age from childhood to elderly. Approximately 90 per cent of all malignant tumors in the ovary are epithelial. Other types are sex cord stromal tumors (6%), germ cell tumors (3%), and tumors of indeterminate histogenesis (1%)[6]. Still the percentages are varied among different age groups.

The etiology of ovarian cancer is poorly understood. Multiple factors are suggested as a contribution to the initiation and progression of the disease. About 1.5-5 per cent of ovarian cancer are estimated as hereditary with some support of an autosomal dominant transmission[1,7]. The hypothesis that ovarian cancer develops from an aberrant repair process of the surface epithelium after a repetitive ovulation cycle, is supported by the protective effect of parity, multiple births, history of breast feeding and oral contraceptive usage[1,8]. Some environmental factors relating to the occurrence of ovarian cancer include talc (magnesium silicate) usage, asbestos exposure, high intake of animal fat and meat[9,10]. However, these are more likely to be epidemiological evidence without strong definite research support. During the past 20 years until now the occurrence of ovarian cancer has continued to rise slowly without any significant change in the survival rate[1].

At present, recent advances in molecular biology have dramatically progressed in revealing a better understanding of the biomolecular events involved in growth regulation and transformation. During the past two decades, studies in the area of oncogenes and tumor-suppressor genes have begun to elucidate the pathogenesis of human cancer[11, 12]. The results offer a strong support for the idea that cancer could develop and become more dangerous primarily because cells in a single lineage accumulate defects in the genes that normally regulate cell proliferation. Changes in the genes can presumably facilitate the ability of a tumor to grow, invade local tissues and establish distant metastasis. Hormones and other factors in the environment and genetically altered cells almost certainly enhance the cellular deregulation[10-12].

Tumor suppressor genes have been postulated to encode proteins that regulate normal growth and suppress neoplastic development[13-15]. Among the 3 well established tumor suppressor genes, retinoblastoma (*RB*), deleted in colorectal cancer (*DCC*) and *p53*, the latter *p53* gene is the most frequently affected and detected in human epithelial ovarian cancer. *p53* gene is 16-20 kb in length, located in the short arm of chromosome 17. Only exons 2-11 of the total 11 exons of this gene, are the coding area which will transcribe into 2.2-2.5 kb mRNA and further translate into a 293 amino-acid length *p53* protein. Almost (90%) mutations in *p53* gene are found in exons 5 to 8 which are involved mainly within codons 132-281[16-20]. Thus, most mutation analyses of *p53* gene have been confined to these exons[17,18]. Approximately 50 per cent of human ovarian cancers contain various mutations in the *p53* gene resulting in the overexpression of the mutation protein[14]. Wild type or natural *p53* protein is a nuclear phosphoprotein of 53 kD with a short half life and present in too small amount of tissue to be detected. Its function is to be responsible for identifying and repairing mistakes made when DNA in a replicating cell is copied[15]. If *p53* gene is deleted or damaged, the mutant *p53* protein can do more than release a brake on cell proliferation. It can also reduce the ability of cells to limit an extra blood vessel growing in the tumor resulting in a tumor growth and early distant metastasis. In addition, the abnormal protein yielded by the altered gene may affect tumor cells in resisting the destructive effects of radiation and chemotherapy[12]. Then

positive *p53* gene mutation tumors are mostly correlated with bad prognosis and early recurrence. Most mutations in *p53* gene found in human cancers seem to be present in the gene spanning codons 132 to 281 (with the hot spots at codons 175, 248 and 273) corresponding to exons 5, 6, 7 and 8[15-18]. Immunolocalization of *p53* protein in cancer tissue is accepted as a rapid, inexpensive and more suitable method for a routine check up for *p53* gene mutation. However, this could not confirm the exact mutation events of the gene, also some detected mutant genes may not overexpress *p53* protein in the cell[12]. The authors then selected the direct molecular approach to analyse the DNA sequences of *p53* gene from exons 5 to 8 in ovarian cancer tissue after surgical removal[21-23]. The identification of various mutations were compared to the histological types, stages, grades and response to treatment of the cancer.

MATERIAL AND METHOD

Subject collection

Thirty patients with 28 histologically proven primary ovarian cancers at various stages, and 2 metastatic tumors of the colon were admitted to Siriraj Hospital between March 1995 and March 1996. They all received surgical treatment followed by combined cisplatinum based, chemotherapy in addition to a complete follow-up for 36 months afterwards. Approval from the Institutional Review Board as well as informed consent from the subjects was obtained for the collection and molecular analysis of their blood and tumor samples. The authors received both fresh and paraffin embedded tissues from all cases. All primary ovarian cancers were 10 serous cystadenocarcinoma, 6 endometrioid, 4 dysgerminoma, 3 mixed cell type, 2 mucinous cystadenocarcinoma, 2 adenocarcinoma and 1 clear cell carcinoma (Table 1).

Paired blood samples were collected for DNA analysis for comparison with the tissue result. All fresh tissues were kept at -80°C before DNA extraction.

Tissue and white blood cell extraction for DNA[21]

Fresh tissue extraction

About 20 mg of fresh tissue was cut into small pieces and homogenized in tris buffer, then digested overnight at 37°C by proteinase K. After stopping the reaction by potassium acetate at 4°C, the genomic DNA was extracted by phenol-chloroform solution and precipitated by absolute ethanol. The precipitation was dissolved in distilled water and kept at 4°C for study.

Paraffin-embedded tissue extraction

Marked cancer area from about 5-10 slides of each 10 μm thick section of paraffin-embedded tissue was scraped and pooled together. The scraped tissue was deparaffinized twice with xylene that yielded cancer tissues. The steps of DNA extraction by phenol-chloroform solution followed by the ethanol precipitation were the same.

The percentage of tumor cells in fresh frozen and paraffin embedded tissues was about 90 per cent *versus* stromal cells and inflammatory cells as estimated by microscopic finding.

WBC DNA extraction

5 ml of EDTA blood was lysed in RCLB lysis buffer. The pellet of WBC was collected and

Table 1. Histological findings of all 28 primary ovarian cancer tissues.

Primary ovarian cancer tissues	Total	% of each type
Serous cystadenocarcinoma	10	36
Endometrioid	6	21
Dysgerminoma	4	14
Mixed cell type (mucinous, serous and endometrioid)	3	11
Mucinous cystadenocarcinoma	2	7
Adenocarcinoma	2	7
Clear cell type	1	4
Total	28	100

digested by proteinase-K in another lysis buffer to release DNA. The step of DNA extraction and precipitation were the same.

p53 gene amplification by PCR and PCR purification(21-23)

Amplification of exons 5 to 8 from fresh ovarian tissue and WBC extract

Exon 5 to exon 8 of *p53* (nt 12965 to nt 14698) was amplified in a reaction mix composed of 50-100 ng of gDNA from fresh ovarian cancer tissues or leucocytes, 10 x PCR buffer (10 mM Tris-HCl, pH 8.3; 1.25 mM $MgCl_2$; 50 mM KCl; 0.02% gelatin), 0.2 mM dNTPs, 20 pmol of S5 and A8 primers, and 1.25 units of Taq DNA polymerase (Promega). The amplification from paraffin embedded tissue was performed identically, except that different primers for each exon were used. The primer sequences and specific product sizes are listed below. The cycling conditions were 30 cycles (2 minutes at 94°C, 1 minute at 58°C and 2.5 minutes at 72°C). As the first step of the PCR reaction, samples were heated to 94°C for 4 minutes. At the end of all PCR runs, a last extension was done at 72°C for 9.5 minutes.

Exon	PCR Primer	Position	Sequence 5' to 3'	Product size (bp)
5-8	S5	12965-12986	CATGTTTGTTTCTTTGCTGCCG	1733
	A8	14698-14677	TGGTGTTGTTGGGCAGTGCTAG	
5	S5	12965-12986	CATGTTTGTTTCTTTGCTGCCG	480
	R6	13445-13426	TTGCAAACCAGACCTCAGGC	
6	F6	13292-13311	CCTCTGATTCCTCACTGATT	243
	A6	13535-13512	GGTCAAATAAGCAGCAGGAGAAAG	
7	S7	13893-13911	GACAGAGCGAGATTCCATC	223
	R7	14116-14097	TCCTGACCTGGAGTCTTCCA	
8	F8	14443-14462	CCTGAGTAGTGGTAATCTAC	255
	A8	14698-14677	TGGTGTTGTTGGGCAGTGCTAG	

Purification of amplified DNA products

The PCR products were fractionated in 1 per cent low melting agarose gel stained with ethidium bromide. Each band was separately cut out of the gel under the UV light and then purified. The excised DNA fragment was then dissolved in 3 times the volume of Qiagen buffer after being incubated at 50°C for 10 minutes. The purification of the amplified DNA was done by passing through a QIA quick column(24) (Qiagen Inc, Valencia, California). After discarding the flow-through solution, the Qiagen bound DNA was eluted by 30 µl of 10 mM Tris-Cl to be kept at -20°C for further study.

p53 gene sequencing

Purified PCR product was run for direct sequencing of exons 5 to 8 of *p53* gene. The sequencing reaction was done by using the Taq Dye DeoxyTM Terminator Cycle Sequencing kit (Perkin Elmer, Applied Biosystems Division, Foster City, USA). The primer sequences for each exon are listed as following.

Exon	Sequencing Primer	Position	Sequence 5' to 3'	Base
5	S5	12965-12986	CATGTTTGTTTCTTTGCTGCCG	22
6	A6	13535-13512	GGTCAAATAAGCAGCAGGAGAAAG	24
7	F7	13986-14005	GTGTTATCTCCTAGGTTGGC	20
7	R7	14116-14097	TCCTGACCTGGAGTCTTCCA	20
8	A8	14698-14677	TGGTGTTGTTGGGCAGTGCTAG	22

Each reaction consisted of 0.1-0.2 μg of purified DNA, 3.2 pmole of primer, 8 μl of terminator ready reaction mix in a total volume of 20 μl. Then the first cycle of denaturation was run at 95°C for 2 minutes followed by 25 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 50°C) and polymerization (4 minutes at 60°C).

Purification of the sequencing product was performed to remove the excess of Taq dye deoxy terminators. The phenol-chloroform extraction was done twice to remove the sequencing product from the sequencing reaction. The upper aqueous layer containing the product was separated and precipitated by adding of 15 μl of 2 M sodium acetate and 300 μl of 100 per cent ethanol. After centrifugation, the pellet of purified product was collected and resuspended in 25 μl of the template suppression reagent (TSR) for further sequencing by an automated DNA sequencer, model ABI 373A DNA sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, USA).

RESULTS

Studied subjects

Twenty-eight pairs of fresh and paraffin embedded ovarian cancer tissues together with their blood samples were used for *p53* gene analysis. After surgery, all patients were given a chemotherapy course of cisplatin and cyclophosphamide. The authors classified the studied patients into 4 groups according to the results of treatment (Table 2). The complete response group (CR), 14 out of 28 (50%) primary ovarian cancer patients were defined as those who had the complete chemotherapy course and stayed without disease longer than 36 months after being diagnosed. The complete response and recurrent group (CR+RC), 7 out of 28 (25%), were those after having completed chemotherapy they stayed without disease until recurrence, usually 5 to 28 months later. The other 2 groups, partial response (PR), 6 out of 28 (21%), and no response (NP), 1 out of 28 (4%) were patients who stayed with a variable amount of cancer during all through the study. Most of the latter 2 groups died soon, usually 5 months or later after being diagnosed (Table 2).

These 4 groups of patients with different responses were found in all stages of the disease. The CR and CR+RC groups were found up to 8 out of 8 cases (100%) in early stage cancer (stage I, II), while it decreased to 13 out of 20 (65%) in late stage disease. However, the overall response rates for CR and CR+RC in all stage primary ovarian cancer were 21 out of 28 cases (75%)

DNA amplification from tissue and WBC extract

All 28 pairs of fresh and paraffin embedded tissues from the ovarian cancer patients together with the paired blood samples were performed for DNA extraction. Then the amplification of *p53* gene (exons 5 to 8) was successfully processed by the method of polymerase chain reaction followed by a purification, to be ready for further sequencing (Fig. 1).

p53 gene (exons 5 to 8) sequencing

Direct DNA sequencing of exons 5 to 8 of *p53* gene was done in all fresh and paraffin

Table 2. Twenty eight primary ovarian cancer patients were classified into 4 groups of various responses to treatment. CR : complete response and stayed without disease more than 36 months, CR+RC : complete response and then recurrent at months 5-28 after treatment, PR : partial response that mostly died after 5 months of treatment, NR : no response, less survival than PR.

Ovarian cancer	Cases	% of each group	Response to treatment (%)			
			CR	CR+RC	PR	NR
Stage I	4	14	4	-	-	-
Stage II	4	14	1	3	-	-
Stage III	16	58	8	4	3	1
Stage IV	4	14	1	-	3	-
Total primary cancer	28	100	14 (50%)	7 (25%)	6 (21%)	1 (4%)

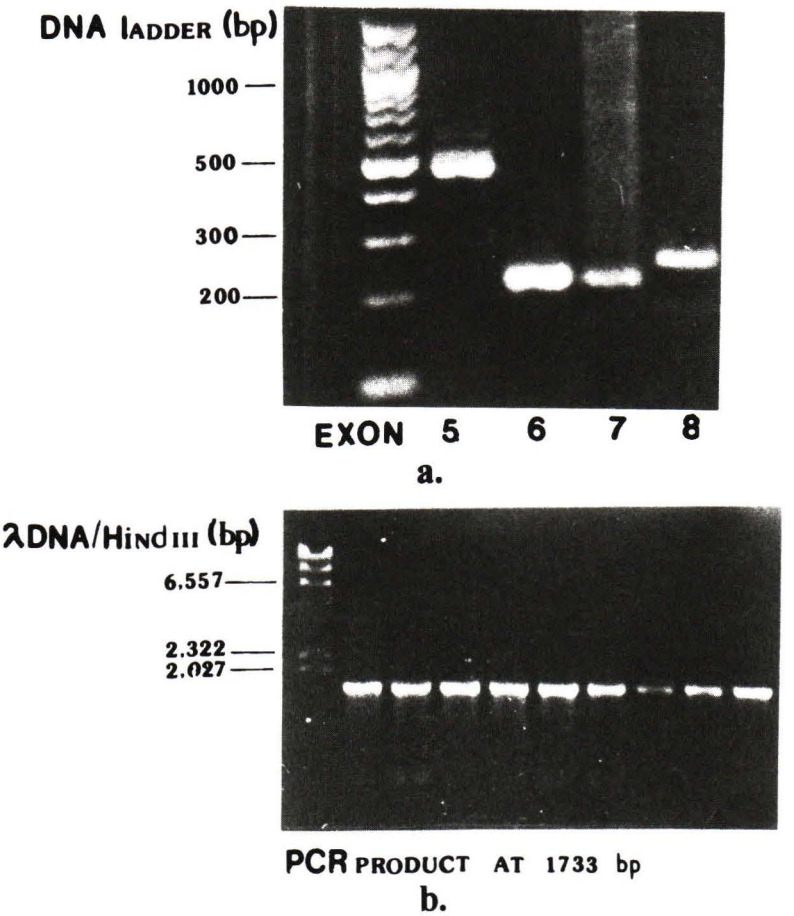

Fig. 1. Electrophoretic bands of amplified *p53* gene from :
a. paraffin embedded tissues of 1 patients showing exon 5 (480 bp), exon 6 (243 bp), exon 7 (223 bp) and exon 8 (255 bp)
b. fresh tissues of 9 patients showing one single band of exons 5 to 8 (1733 bp).

embedded tissues including the paired blood samples. The authors could detect 5 mutations in only exon 5 of the *p53* gene from all of the tissue extracts. Four point mutations and 1 insertion mutation were found in 4 cases of complete response (80%), and 1 case of partial response (20%).

When comparing all patients either with or without *p53* gene mutations, there were no different responses to treatment either at early or late stages (Table 4). Every individual in both groups in the early stages (stage I+II) showed response to treatment (CR, CR+RC). For the late stage groups (stage III+IV) 13 out of 20 cases responded to treatment while 7 cases did not.

DISSCUSSION

Many studies have reported the benefit of paraffin sections for DNA study since they could preserve the morphology of cancer cells better than the frozen tissue sections. However, the yield of cancer cells scraped from paraffin sections was very minute and also contained inhibitors for most enzy-

Table 3. Details of 5 ovarian cancer patients with *p53* gene mutations. M : married, S : single, PR : partial response, CR : complete response.

Patient	Age (y)	S/M	Tissue no.	Histological classification	*p53* mutation			Response to treatment
					Nucleotide	Amino acid codon	Type of mutation	
1.	56	M	3 US	Endometrioid stage IV grade -	C 13059 T	Ser 127 phe	Heterozygous	PR (stay with disease)
2.	37	S	54 S	Endometrioid stage I grade I	A 13232 G	Ser 185 gly	Heterozygous	CR
3.	30	M	28 L	Endometrioid stage I grade I	G 13235 C	Asp 186 his	Heterozygous	CR
4.	40	M	10 R	Serous cystadenocarcinoma stage III grade I	G 13235 C	Asp 186 his	Heterozygous	CR
5.	45	S	35 L	Serous cystadenocarcinoma stage III grade I	ATG13186AT$\underline{A}$G	Met 169 frameshift	Homozygous	CR

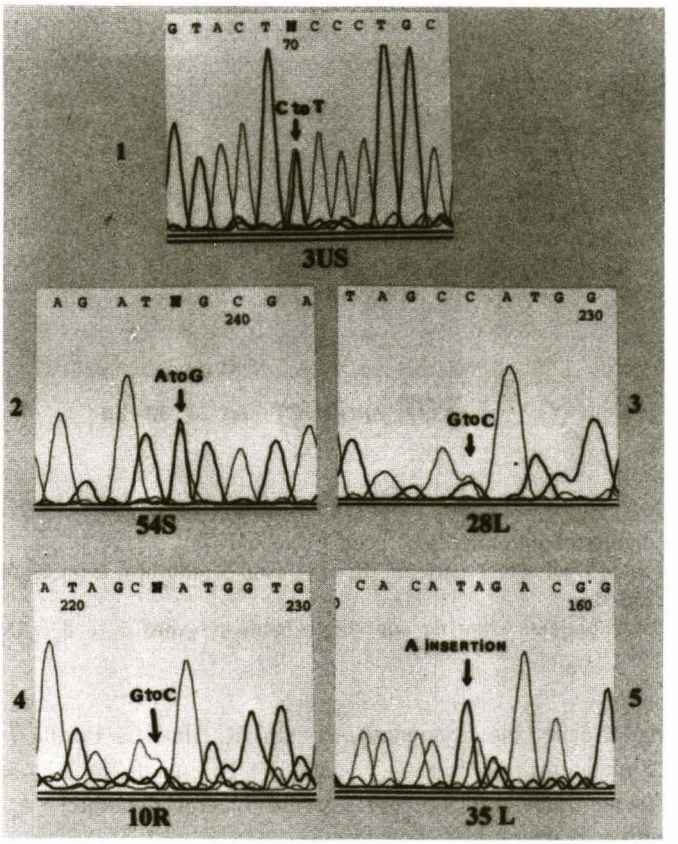

Fig. 2. ***p53*** **gene mutation in exon 5 of 5 patients, cases 3US (1), 54S (2), 28L (3), 10R (4) and 35L (5) with the point mutations at nucleotides 13059 (C→T : ser 127 phe), 13232 (A→G : ser 185 gly), 13235 (G→C : asp 186 his), 13235 (G→C : asp 186 his) and the insertion at nucleotide 13186 (TGÆT$\underline{A}$G : frameshift mutation), respectively.**

Table 4. Ovarian cancer patients with and without *p53* mutation show no different responses to treatment either in early stage or late stage groups. Response groups include CR, and CR+RC, while no response groups are PR and NR.

Ovarian cancer	Total (28) cases		*p53* Mutation (5)		No Mutation (23)	
	Response (21)	No Response (7)	Response (4)	No Response (1)	Response (17)	No Response (6)
Early stage I + II	8/8	-	2/2	-	6/6	-
Late stage III + IV	13/20	7/20	2/3	1/3	11/17	6/17

matic steps in PCR and DNA sequencing[24,25]. The successful rate of PCR and sequencing from paraffin preserved tissues was low and required complicated techniques. The authors succeeded in sequencing the *p53* gene in 28 out of 38 patients (74%).

As from direct DNA sequencing of exons 5 to 8 of *p53* gene the authors could detect 5 mutations in only exon 5. The result was the same as many reports that the exon 5 was the hottest region, with 71 per cent detection of all exons[16-19,25]. However, it is possible that mutations in Thai patients with ovarian cancer may locate in other exons or regions of *p53* gene. Unfortunately, preliminary study was not performed on those areas.

The types of the detected mutations, point and insertion mutations found in codons 127, 185, 186 and 169, in *p53* gene from 5 epithelial ovarian cancers (3 endometrioid and 2 serous cystadenocarcinoma) were similar to the study of Kim et al[26]. Moreover, the present data showed no mutation in 4 cases of dysgerminoma which was the same as a report by Liu et al[27]. This could support that the pathogenesis of this type of germ cell cancer might not associate with the *p53* gene defect. The nucleotide changes observed in the present study are likely to be mutations rather than DNA polymorphisms since they are all in conserved regions. In addition, some reported data have shown that 86 per cent of *p53* mutations are located between codons 120 and 290[28-30], where the mutations in the present study were found. Work is now underway in our laboratory in order to clarify whether these changes are mutations or DNA polymorphisms.

Many papers reported that *p53* gene mutations were mostly found in late stage ovarian cancer and associated with bad prognosis. However, a number of studies have shown no association between *p53* gene mutations and poor survival outcome (31-33). From the present study, the authors found *p53* mutations in 5 out of 28 cases (18%) in primary ovarian cancer of Thai patients. This finding was lower than the average of about 50 per cent from many reported data[13,25-27]. The mutated genes were detected in all stages of epithelial ovarian cancer, but not in metastatic cancer. Although only 5 patients with mutations seemed to be inadequate for statistical analysis of responsiveness to treatment, 4 out of the 5 cases had a complete response to treatment and stayed without disease longer than 36 months. This might be explained partly that the histological types of cancer in those 5 patients had good response to chemotherapy.

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การกลายพันธุ์ของยีน *p53* ในมะเร็งรังไข่ของคนไทย

นิโลบล เนื่องตัน, พ.บ., สมชาย เนื่องตัน, พ.บ.**,*
*ชัยรัตน์ ลีลาพัฒนดิษฐ์, พ.บ.**, จงดี แดงรัตน์, วท.ม.**, รุ่งทิพย์ สร้อยอัมพรกุล, วท.ม.**

วัตถุประสงค์ : เพื่อศึกษาลักษณะการกลายพันธุ์ของยีน *p53* ในมะเร็งรังไข่ของคนไทย และนำมาเปรียบเทียบกับการตรวจพบทางพยาธิวิทยาและทางคลินิก

วัสดุและวิธีการ : นำชิ้นเนื้อมะเร็งรังไข่ชนิดปฐมภูมิ 28 รายและชนิดที่กระจายจากมะเร็งของอวัยวะอื่น อีก 2 ราย มาวิเคราะห์ ลำดับเบส บน DNA (ในช่วง exon 5–8) ของยีน *p53* ซึ่งเป็นช่วงที่พบความผิดปกติได้บ่อยที่สุด จากนั้นจึงนำการกลายพันธุ์ที่ตรวจพบได้ในชิ้นเนื้อแต่ละรายมาหาความสัมพันธ์เปรียบเทียบกับลักษณะทางพยาธิวิทยาและผลการรักษาในผู้ป่วยตั้งแต่แรกจนถึง 36 เดือนต่อมา

ผลการศึกษา : พบการกลายพันธุ์ของยีน *p53* จำนวน 5 ราย จากผู้ป่วยมะเร็งรังไข่ 28 ราย (ร้อยละ 18) เป็นชนิด insertion 1 ราย และชนิด point 5 ราย ไม่พบการกลายพันธุ์จากการตรวจเลือดในผู้ป่วยทั้งหมดนี้ สำหรับลักษณะทางพยาธิวิทยาของมะเร็งที่ตรวจพบการกลายพันธ์ เป็นชนิด endometrioid 3 ราย และ serous cystadenocarcinoma 2 ราย โดยมีระยะของโรคแตกต่างกันตั้งแต่ระยะที่ I–IV อีกทั้งมีผลการตอบสนองต่อการรักษาอยู่ในเกณฑ์ดีเต็มที่ 4 รายจาก ผู้ป่วยกลุ่มนี้ 5 ราย (ร้อยละ 80) การประเมินทำภายหลังการรักษาด้วยการผ่าตัดตามด้วยการให้สารเคมีบำบัดครบชุดและติดตามอาการต่อมาอีก 36 เดือน ในขณะที่ผลการรักษาเช่นเดียวกันนี้พบได้ในผู้ป่วย 14 ราย จากผู้ป่วยมะเร็งรังไข่ทั้งหมด 28 ราย (ร้อยละ 50)

สรุป : ผู้ป่วยมะเร็งรังไข่ 5 ราย ที่มีการกลายพันธุ์ของยีน *p53* เมื่อได้รับการรักษาตามเกณฑ์มาตรฐานครบถ้วน มีผลตอบสนองต่อการรักษาเช่นเดียวกับผู้ป่วยมะเร็งรังไข่ทั่วไป ปัจจัยการตอบสนองต่อการรักษาของมะเร็งรังไข่ในคนไทยยังคงขึ้นกับระยะของโรค เกรดของเนื้อเยื่อ และลักษณะทางพยาธิวิทยา

คำสำคัญ : มะเร็งรังไข่, การกลายพันธ์ของยีน *p53*, ข้อมูลการเรียงดีเอ็นเอ, การพยากรณ์โรค

นิโลบล เนื่องตัน, สมชาย เนื่องตัน,
ชัยรัตน์ ลีลาพัฒนดิษฐ์, จงดี แดงรัตน์, รุ่งทิพย์ สร้อยอัมพรกุล
จดหมายเหตุทางแพทย์ ฯ 2545; 85: 658–667

* ภาควิชาชีวเคมี,
** ภาควิชาสูติศาสตร์–นรีเวชวิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพ ฯ 10700