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p53 Suppressor Gene Mutations in Ovarian Cancer

Thongbliew Prempree, MD, PhD*
Rumpa Amornmarn, MD*
Robert C Nuss, MD**
Guy I Benrubi, MD**
Boniface Ndubisi, MD**

**Department of Radiation Oncology, **Department of Obstetrics and Gynecology, University of Florida, University Medical Center Jacksonville, Florida, USA.*

PRECIS : *Mutations of p53 gene have been discovered in a wide variety of human cancers. We studied the mutation of p53 gene in 40 consecutive ovarian cancers and found p53 proteins overexpression by Western Transfer Method to be at the rate of 95 per cent (38/40), while the presence of p53 mutation by DNA Sequencing was at the rate of 78 per cent (31/40). DNA Sequencing should be done in all cases without exception to be able to capture all mutations.*

Abstract

Mutations of p53 suppressor gene have been discovered in a wide variety of human cancer ranging from most common, such as basal cell cancers to the least common, such as soft tissue sarcoma. It is believed that the accumulation of mutations, exogenous or endogenous in the genome of cells can lead to deregulation of cell growth and finally development of cancer.

We studied the p53 mutations in 40 consecutive ovarian cancers by using Immunoblotting (Western Transfer Method) to detect mutant p53 protein in ovarian cancer cells and DNA sequencing technique to identify mutation in p53 gene.

It was discovered that overexpression of p53 protein was at the rate of 38/40 = 95 per cent and the presence of mutations in p53 gene was 31/40 = 78 per cent. Even though the correlation between p53 overexpression and presence of mutation was not perfect, the data clearly showed that the p53 mutation was found in most ovarian cancer and perhaps represented somatic mutation leading to this cancer. A total of 41 mutations and one deletion were discovered in 31 cases, more than $\frac{1}{2}$ were of Transition mutation type and less than $\frac{1}{2}$ were of Transversion type of mutations.

p53 Mutation was detected in ovarian cancer from early to late stage. It is thought perhaps that p53 mutation should be viewed as initial step of carcinogenesis rather than late event. One half of the p53 mutations may arise from spontaneous mutation, yet the other one half may arise from interaction of carcinogens with DNA.

Key Words: p53 mutation in ovarian cancer; p53 overexpression; DNA sequence; ovarian cancer; p53 suppressor gene.

p53 Suppressor gene encodes a nuclear phosphoprotein present in low levels in normal human cells.[1,2] Wild-type p53 tumor suppressor gene plays a central role in the maintenance of normal cell growth, differentiation and programmed cell death (apoptosis).[1,3] It is thought perhaps that loss of normal function of this suppressor gene could cause the cell to lose its self control, therefore allowing the abnormal growth of the cell to occur.[1,4-7] Since the discovery of p53 suppressor gene and many years of extensive research, mutations of p53 genes have been discovered in a wide variety of human cancers, from the more common one such as basal cell skin cancer,[8] colon,[9,10] breast,[11,12] lung,[13] brain,[14,15] to the less common such as hepatocellular cancer,[16,17] and soft tissue sarcomas.[15] Epithelial ovarian cancers have also been shown, by many researchers, to exhibit loss of normally functioning p53 gene with mutations.[10,15,18-28]

While continuing attempts are being made to improve early diagnosis and treatment of ovarian cancer, many significant gains have been made in the knowledge of the molecular mechanisms of the carcinogenesis of this malignancy.

In the present study we used Western Transfer Method to identify mutant p53 protein and further identify p53 mutation in 40 consecutive ovarian cancers and compiled the specific types of base substitution of p53 gene from Exon 4 to Exon 8.

Materials and Methods

Clinical materials

Fresh tumor tissue was collected from 40 patients following surgical debulking and divided into two portions; one for DNA study; the other for protein study. In the event that ascitic fluid was collected, it was processed immediately by centrifuging to obtain cell pellet for both protein and DNA studies. These patients were treated at University Medical Center, from 1994 to the early part of 1996. Their ages ranged from 24 to 86 years, with a median age of 63 and an average age of 60.6 years old. There were 37 cases of papillary serous cystadenocarcinoma and 3 cases of mucinous cystadenocarcinoma.

Protein study/Western Trasfer analysis[29]

A) Sample Preparation:

Tissue samples were washed with 1x phosphate buffered saline, minced by scalpel into smallest portions and disrupted by sonication in 200 µl lysis buffer (1% sodium dodecyl sulfate SDS and 10 mM Tris HCl pH7.4). The homogenates were brought to the boiling point by microwave for 10-15 seconds. Lysates were spun down and the supernatant fluid was removed and measured for protein concentration. The sample was diluted by loading buffer to yield 10 µg per 1 µl, per lane for electrophoresis.

B) Polyacrylamide Gel Electrophoresis[29,30]

Under denaturing conditions, SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) proteins were separated based on molecular size as they moved through the polyacrylamide gel matrix toward anode (Laemmli system).[30] Tris/glycine polyacrylamide 8-16% gradient gel were used and run at 125 volts/30-35 mA for $1\frac{1}{2}$ hours.

C) Electrophoretic Transfer (Blotting)

After protein separation by electrophoresis, the transfer or blotting involved the trasfer of resolved proteins from a gel onto the surface of a membrane. As a rule, following electrophoresis, the protein molecule of interest was trapped within the gel matrix, making the study difficult. When the molecule was transferred to the membrane, (we use PVDF-Polyvinylidene Difluoride), the protein molecule was bound to the membrane surface and became accessible to the molecular probe and detection device. We used electrotransfer technique (Semiphor 70 Hoefer) for our study.

D) Blocking and Antibody Treatment

After the completion of electro-transfer, the blot was removed from the transfer apparatus, and immediately placed into the blocking buffer (1% BSA in 10mM Tris pH 7.5, 50 mM NaCl and 0.1% Tween 20). The blot was allowed to stay at 4°C for overnight. After decanting blocking buffers, anti-p53 monoclonal antibody (Transduction Lab or Oncogene Science) was added to the blot with continuous agitation for 1 hour at room temperature.

E) Incubation with The Second Antibody

Primary antibody solution was decanted and wash buffer (10 mM Tris pH 7.5, 100 mM NaCl 0.1% Tween 20) was added to wash the blot for 30 minutes with agitation. The wash buffer was changed every 5 minutes. After the last change of the wash buffer, the second antibody/Anti-mouse Immunolobulin with Alkaline Phosphatase Conjugate (Sigma Immuno

Chemicals) was added and allowed to incubate at room temperature for 1 hour with mild agitation to be sure that the second antibody was interacting well with the blot.

F) Substrate Incubation

The antibody conjugate was decanted and wash buffer (10 mM Tris pH 7.5, 100 mM NaCl 0.1% Tween 20) was added for blot washing for 30 minutes with agitation. The wash buffer was changed every 5 minutes. After the last wash, the blot was transferred into a plastic bag containing ECL reagent (Amersham) for chemiluminescent detection. The substrate for Alkaline Phosphatase was Dioxetane. The ECL reagent interaction with the Alkaline Phosphatase conjugate resulted in light production. Kodak X-OMAT AR film was applied to the blot until satisfactory exposure was achieved, usually about 1-2 hours. After exposure, the film was developed with the usual technique and the results interpreted (Figure 1).

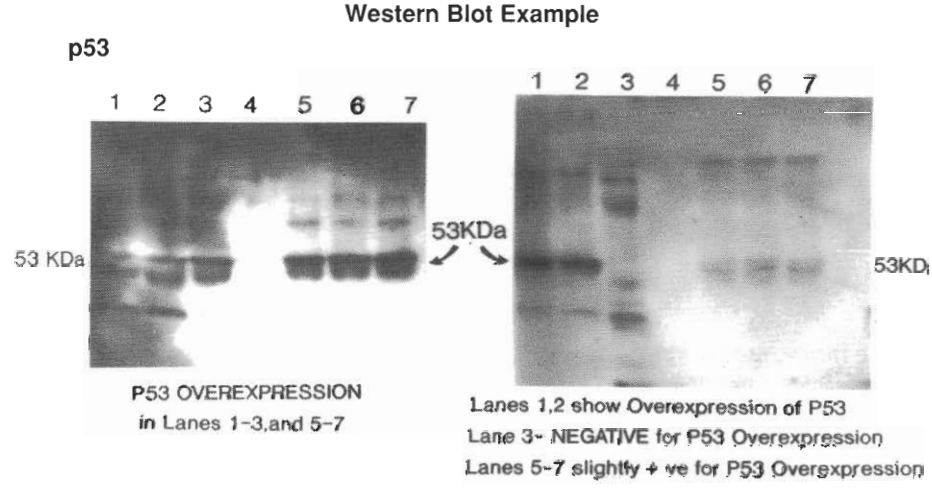

Fig. 1 Example of Western Transfer (Blot) Technique to detect mutant p53 protein by chemiluminescent method. p53 protein has molecular weight of 53 kDa (Kilodalton). Left panel, all six lanes 1-3 and 5-7 are positive for overexpression. Right panel, lanes 1 and 2 are positive, lane 3 is negative. Lanes 5, 6, and 7 were interpreted as negative for overexpression in spite of faint band shown here.

Table 1 Primer Sequence

Exon		Primer Sequence	Size of Amplified Fragment (bp)
4	Forward	5'- CAC CCA TCT ACA GTC CCC CTT GC-3'	307
	Reverse	5'- CTC AGG GCA ACT GAC CGT GCA AG-3'	
5	Forward	5'- CTC TTC CTG CAG TAC TCC CCT GC-3'	211
	Reverse	5'- GCC CCA GCT GCT CAC CAT CGC TA-3'	
6	Forward	5'- GAT TGC TCT TAG GTC TGG CCC CTC-3'	185
	Reverse	5'- GGC CAC TGA CAA CCA CCC TTA ACC-3'	
7	Forward	5'- GTG TTG TCT CCT AGG TTG GCT CTG-3'	139
	Reverse	5'- CAA GTG GCT CCT GAC CTG GAG TC-3'	
8	Forward	5'- ACC TGA TTT CCT TAC TGC CTC TGG C-3'	200
	Reverse	5'- GTC CTG CTT GCT TAC CTC GCT TAG T-3'	
9	Forward	5'- GCC TCT TTC CTA GCA CTG CCC AAC-3'	102
	Reverse	5'-CCC AAG ACT TAG TAC CTG AAG GGT G-3'	

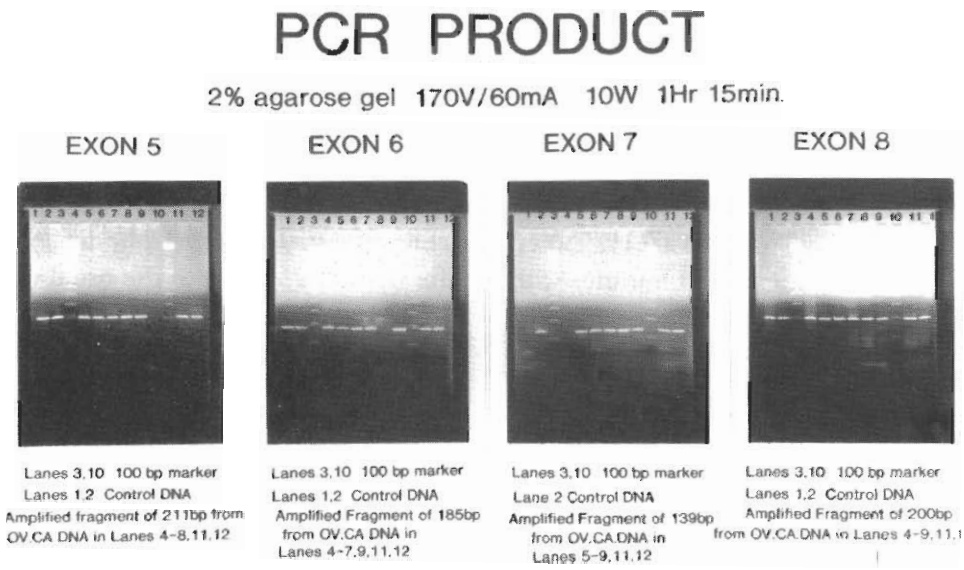

Fig. 2 Example of PCR products run on 2 per cent agarose gel from Exon 5, 6, 7 and 8.

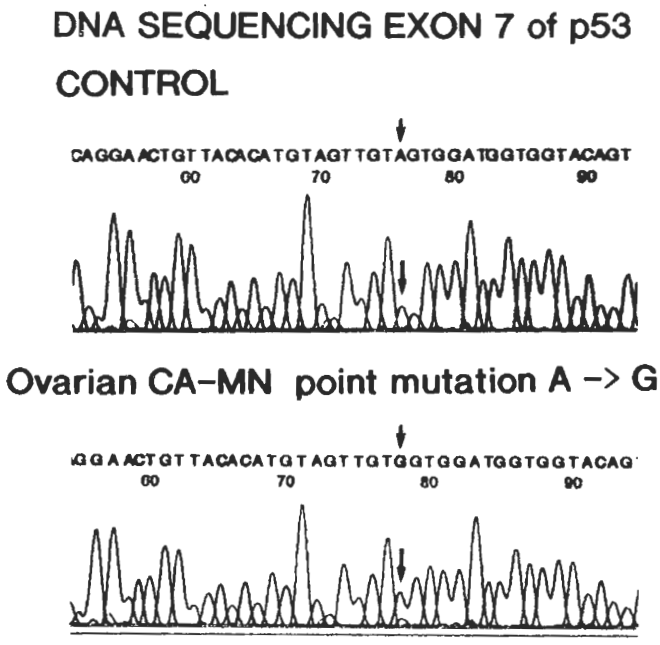

Fig. 3 Partial sequence of p53/Exon 7 of both control DNA and Ovarian Cancer/MN 62 DNA. Observe the base change from A in control of G in Ovarian Cancer DNA.

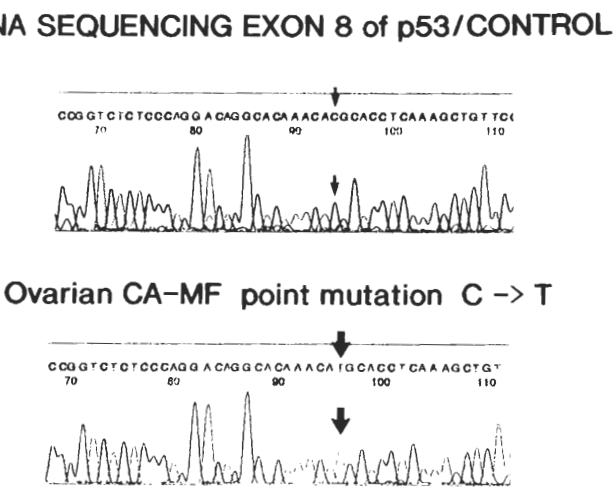

Fig. 4 Partial sequence of p53/Exon 8 of both control and Ovarian Cancer/MF 79. As shown, base C in control sequence changes to T in Ovarian Cancer DNA of patient MF 79.

DNA Study

A) DNA preparation

Using standard techniques, we extracted and purified genomic DNA from ovarian cancers and control DNA from normal individual or adjacent normal tissue that was determined histologically to be free from cancer.[31]

B) PCR (Polymerase Chain Reaction)

To Search for gene aberrations by PCR/DNA Sequencing Method, the coding regions of the p53 gene particularly from Exon 4 to Exon 9 were amplified individually into DNA fragments with the help of two sets of primers (forward and reverse) for each exon (Table 1). Each reaction mixture 50 µl contained 200 ng of tumor DNA, 1.5 mM of $MgCl_2$, dNTP mix of 0.2 mM, Taq DNA polymerase (Promega or Boehringer Mannheim) 2.0 U, and forward and reverse primers (0.4 µM each). Amplification was carried out in Perkin

Elmer Thermal Cycler. Thirty five cycles of 30 seconds at 95°C, 45 seconds at 66°C and 90 seconds at 72°C were used and followed by 7 minutes at 72°C, and finally at 4°C for wait cycle. The PCR product was tested for amplified fragments in 2 per cent agarose containing Ethidium Bromide. A clean single band should result as shown in Figure 2. The PCR product was purified by MicroSpin Sephacryl HR column chromatography (S-400 HR from Pharmacia Biotech) before DNA sequencing.

C) DNA Sequencing

The enzymatic method of Sanger et al[32] was used in both manual and automated DNA Sequencing Techniques. The Sanger method is based on the use of specific terminators of DNA chain elongation: 2',3', dideoxynucleoside-5'-triphosphate (ddNTP). These deoxynucleoside triphosphate analogues can be incorporated by a DNA polymerase (we use taq DNA polymerase from Perkin Elmer or Amersham) into a growing DNA chain through their 5'-triphosphate groups. However, because these analogues lack a hydroxyl group at the 3' position of deoxyribose sugar, they can not form phosphodiester bonds with the next incoming deoxynucleoside-5' triphosphate (dNTP) and the chain extension terminates whenever that analogue is incorporated. To obtain sequence data, one extension reaction must be run for each of the four ddNTPs. We used AmpliCycle Sequencing Kit, either from Perkin Elmer or Amersham for our experiments.

The strategy for Thermal Cycle Sequencing involved the removal of leftover primers from the first PCR experiment (we used S-400 HR MicroSpin Sephacryl Column Chromatography). The amplified DNA fragments from Exon 4 to 9 of p53 were used as starting material for the second amplification with only one of the two PCR primers and ddNTPs in the reaction. During the thermal cycling the thermostable polymerase used one strand of the amplified DNA fragment as a sequencing template. Because many samples were prepared for DNA sequencing from all amplified fragments of various exon of p53, it became necessary to use automated DNA Sequencing Techniques extensively. The method was modified from Sanger et al technique.[32] DNA sequencing was performed by fluorescent dye terminator labeling method using ABI Prism Dye Terminator Cycle Sequencing Ready reaction Kit with Ampli Taq DNA Polymerase FS. Sequencing reactions were analyzed on an ABI 373A DNA Sequencer. The data of DNA sequences were analyzed by MacVector software version 5.0 from IBI. Figures 3 and 4 show examples of the automated DNA sequencing data.

Results

Pathologic study

The pathologic feature and tumor grade of the tumors were determined according to FIGO (International Federation of Gynecology and Obstetrics) Classification.[33] Of 40 ovarian cancers studied, 2 tumors were classified as stage I, grade I; 2 tumors as stage II, grade I and 36 tumors as stage III with 31 grade 3 and 5 grade 2.

Overexpression of p53 protein

Intense band formation with molecular weight of 53 kilodalton (kDa) from the blot after sufficient exposure to the x-ray film, indicative of overexpression of p53 protein, was shown in Figure 1. The left panel showed positive overexpression on lanes 1-3 and 5-7. Likewise, on the right panel, lanes 1 and 2 demonstrated overexpression while lane 3 showed no intense band formation at 53 kDa position, indicative of no mutant protein present to interact with anti-p53/ negative overexpression. On lanes 5-7 a faint band at 53 kDa was seen but our interpretation was negative for p53 overexpression.

The data of p53 overexpression were shown in Table 2. Most of our ovarian cancers were stage III (36 cases). Only 2 cases were in stage II and 2 were in stage I disease. Thirty eight cases showed overexpression of p53 protein (38/40 = 95%) and one case of stage I and one case of stage III showed negative overexpression. We examined the relationship between p53 overexpression and tumor grade and histologic subtypes and found no correlation between them (Table 3).

Table 2 Ovarian cancers p53 overexpression by Western Transfer method.

Total Number of Patients	Overexpression	Per cent
40	38	95

Table 3 Ovarian cancer with p53 mutation.

Code	Path/Grade	Stage	p53 Protein Overexpression	Exon	Codon	Base Changes	Amino Acids	Survival	
EJ49	AdenoCA/G3	III	+	4	72	CGC → CCC	ARG → PRO	ED	1 year
JR64	Mucinous Cyst AdenoCA	III	+	5	136	CAA → CTA	GLN → LEU	ED	1 year
MF79	Serious AdenoCA/G2	III	+	5	140	ACC → AAC	THR → ASN	DOD	2 years
EW77	Mucinous Cyst AdenoCA	III	+	5	141	TGC → TGG	CYS → TRP	ED	1 year
MF79	Serous AdenoCA/G2	III	+	5	148	GAT → GAA	ASP → GLU	DOD	2 years
AL29	Serous AdenoCA/G2	II	+	5	175	CGC → CAC	ARG → HIS	DOD	1 year
VC64	Serous AdenoCA/G3	III	+	5	175	CGC → CAC	ARG → HIS	ED	1 year
MF79	Serous AdenoCA/G2	III	+	5	184	GAT → GAA	ASP → GLU	DOD	2 years
EC59	AdenoCA/G3	III	+	6	213	CGA → CAA	ARG → GLN	ED	1 year
LL29	SerousAdenoCA/G1	II	+	6	213	CGA → CAA	ARG → GLN	NED	3 years
LB77	SerousAdenoCA/G3	III	+	6	213	CGA → CAA	ARG → GLN	ED	1 year
MN62	Serous AdenoCA/G3	III	-	7	234	TAC → CAC	TYR → HIS	NED	2 years
HW50	AdenoCA/G3	III	+	7	234	TAC → CAC	TYR → HIS	NED	3 years
GH42	AdenoCA/G3	III	+	7	236	TAC → GAC	TYR → ASP	DOD	1 year
KL86	Serous AdenoCA/G1	I	+	7	241	TCC → GCC	SER → ALA	NED	2 years
GC58	Serous AdenoCA/G3	III	+	7	245	GGC → GAC	GLY → ASP	ED	1 year
MO73	Serous AdenoCA/G3	III	+	8	263	AAT → CAT	ASN → HIS	ED	1 year
MR63	AdenoCA/G3	III	+	8	263	AAT → CAT	ASN → HIS	ED	2 years
DB68	Serous AdenoCA/G3	III	+	8	272	GTG → ATG	VAL → MET	DOD	1 year
BA57	AdenoCA/G3	III	+	8	272	GTG → ATG	VAL → MET	NED	3 years
MF79	Serous AdenoCA/G2	III	+	8	273	CGT → CAT	ARG → HIS	DOD	2 years
SD68	AdenoCA/G3	III	+	8	273	CGT → CTT	ARG → LEU	ED	1 year
MR63	AdenoCA/G3	III	+	8	282	CGG → TGG	ARG → TRP	ED	2 years
EJ49	AdenoCA/G3	III	+	8	284	ACA → TCA	THR → SER	ED	1 year
BL73	AdenoCA/G3	III	+	8	286	GAA → GTA	GLU → VAL	ED	1 year
DS57	AdenoCA/G3	III	+	8	287	GAG → AAG	GLU → LYS	NED	2 years
LH58	Serous AdenoCA/G3	III	+	8	292	AAA → ACA	LYS → THR	ED	1 year
EC59	AdenoCA/G3	III	+	8	296	CAC → CTC	HIS → LEU	DOD	1 year
GC58	Serous AdenoCA/G3	III	+	8	298	GAGC → GAC (deletion)	Frameshift	ED	1 year
DB72	Serous AdenoCA/G3	III	+	8	298	GAG → GAC	GLU → ASP	DOD	2 years
ML70	Serous AdenoCA/G3	III	+	8	298	GAG → AAG	GLU → LYS	DOD	1 year
MO73	Serous AdenoCA/G3	III	+	8	298	GAG → GAC	GLU → ASP	ED	1 year
BB69	Serous AdenoCA/G2	III	+	8	306	CGA → TCA	ARG → SER	ED	2 years
JA71	Serous AdenoCA/G3	III	+	8	306	CGA → TCA	ARG → SER	DOD	1 year

ED = Evidence of Disease (Alive)
DOD = Died of Cancer
NED = Alive with No Evidence of Disease

Direct sequencing and mutation study

Our strategy to perform thermal cycle sequencing of amplified fragments was to include Exon 5 to Exon 8 for all samples of DNA obtained from 40 consecutive ovarian cancers. For those that did not demonstrate any mutations in Exon 5 to Exon 8, the study was extended to cover Exon 4 and Exon 9 as well. If mutation was found in one of the Exons 5-8, the study was not extended to Exon 4 or 9, unless mutation was not clearly seen.

The analysis of DNA sequence was focused on the coding region of these exons. Base change for example, in figure 3, was clearly seen from A in control group to B in ovarian cancer DNA of Exon 7. Base change in Figure 4, was also obvious in control from C to T of patient tumor DNA/MF and Exon 8.

p53 Gene analysis

The aberrations of p53 gene identified by DNA Sequence Analysis were found in 31 of 40 ovarian cancers (78%). All except one aberration were point mutation (Table 3). There were 7 cases that had more than one mutation in their p53 gene. For example, case MF 72 (grade 2) had a mutation at Codon 231 in Exon 7 (ACC→ACT, Transition) and another mutation at Codon 273 in Exon 8 (CGT→CAT, Transition) resulting in changing Arginine to Histidine at Codon 273. Case MO 73 (grade 3) had 2 mutations, one at Codon 263 in Exon 8 (ATT→CAT, Transversion) changing Asparagine to Histidine; the other at Codon 298 in Exon 8 (GAG→GAC, Transversion) changing Glutamic Acid to Aspartic acid. Case MR 63 had 2 mutations in Exon 8; at Codon 263 (AAT→CAT, Transversion) and

Table 4 Ovarian cancer with silent p53 mutation.

Code	Path/Grade	Stage	p53 Protein Overexpression	Exon	Codon	Base Changes	Amino Acids	Survival
LL29	Serous AdenoCA/G1	II	+	5	130	CTC → CTG	LEU → LEU	NED 3 years
OF55	Serous AdenoCA/G2	III	+	5	179	CAT → CAC	HIS → HIS	ED 2 years
VA83	Serous AdenoCA/G3	III	+	6	213	CGA → CGG	ARG → ARG	DOD 1 year
EC59	AdenoCA/G3	III	+	6	221	GAA → GAG	GLU → GLU	DOD 1 year
GS25	Serous AdenoCA/G3	III	+	6	221	GAA → GAG	GLU → GLU	ED 3 years
MF79	Serous AdenoCA/G2	III	+	7	231	ACC → ACT	THR → THR	DOD 2 years
JC56	Serous AdenoCA/G3	III	+	7	238	TGT → TGC	CYS → CYS	ED 2 years
EN61	Serous AdenoCA/G3	III	+	7	248	CGG → CGT	ARG → ARG	ED 1 year

ED = Evidence of Disease (Alive)
DOD = Died of Cancer
NED = Alive with No Evidence of Disease

Table 5 Ovarian cancer site of mutation and base changes.

Exon	Total Mutations	Mutation at G:C			Mutation at A:T		
		→ A:T	→ T:A	→ C:G	→ T:A	→ G:C	→ C:G
4	1	0	0	1	0	0	0
5	9	2	1	2	3	1	0
6	6	3	0	0	0	3	0
7	8	3	1	0	0	2	2
8	17	8	1	2	3	0	3
Total	**41**	**16**	**3**	**5**	**6**	**6**	**5**

at Codon 282 (CGG→TGG Transition). Case GC58 had a point mutation at Codon 245 Exon 7 (GGC→GAC Transition) and a microdeletion at Codon 298 (GAGC→GAC) Exon 8 as well.

Silent mutation

Table 4 showed 8 cases of ovarian cancer with p53 point mutation at one of their bases in a codon, but this did not result in amino acids changes. Interestingly enough, all cases with silent mutation did have p53 protein overexpression. The silent mutations in our study occurred at the rate of 8/41=19.5 per cent.

Table 6 Transition and transversion.

Type of Base Substitution	# Base Substitution	Per cent
Transition:		
G:C → A:T	16	39
A:T → G:C	6	15
Total	**22**	**54**
Transversion:		
G:C → T:A	3	7
G:C → C:G	5	12
A:T → T:A	6	15
A:T → C:G	5	12
Total	**19**	**46**
TOTAL	**41**	**100**

Table 7 Point mutation of p53 in ovarian cancers of various clinical stage.

Clinical Stage	No. of Cases	Cases with Point Mutations (%)	
I	2	1	50
II	1	2	100
III	36	28	78
Total	**40**	**31**	**78**

Site of mutation and base changes (Table 5)

Table 5 compiled type of mutations from Exon 4 to Exon 8. As shown, mutations were divided into two main groups; mutation at G:C and mutation at A:T. For each and every exon, mutations at G:C were subdivided into →A:T, →T:A and →C:G. Likewise, mutations at A:T were subdivided into → T:A, → G:C and → C:G. All mutations at G:C basepair were 24/41 = 59 per cent. All mutations at A:T basepair were 17/41 = 41 per cent. Of 41 total mutations studied, transition type mutation occurred at G:C → A:T = 16 and A:T → G:C = 6, totaling 22 mutations. Transversion mutations G:C → T:A, G:C → C:G, A:T → T:A and A:T → C:G were 3+5+6+5 = 19 mutations (Table 6).

CpG mutation

The data from Tables 3 and 4 showed many CpG mutations from Exon 4 to Exon 8. The data showed one CpG mutation occurred at Exon 4/codon 4; 3 CpG mutations at Exon 5 codons 141, 175 and 175; 4 CpG mutations at Exon 6 codons 213, 213, 213 and 213; 2 CpG mutations at Exon 7, codon 245 and 248. There were 5 CpG mutations at Exon 8, codon 273, 273 282, 306 and 306. All total, 15 CpG mutations (15/41 = 37%) occurred in ovarian cancer.

p53 mutations at various stages and survival

Table 7 showed evidence of p53 mutation by DNA sequencing method at various stages of disease. As seen, mutation was discovered in one of two of stage I and 2 of 2 of stage II disease. Most of our tissue samples were from stage III disease with 28 of 36 (78%) with

Table 8 Ovarian cancer p53 mutation and survival.

Patient	Stage/Grade	Exon/Codon	Mutation Event	Survival
KL86	I/G1	7/241	AT → CG (Transversion)	NED 2 years
LL29	III/G1	6/213	GC → AT (Transition)	NED 3 years
MN62	III/G3	7/234	AT → GC (Transition)	NED 2 years
HW50	III/G3	7/234	AT → GC (Transition)	NED 3 years
BA57	III/G3	8/272	GC → AT (Transition)	NED 3 years
DS57	III/G3	8/287	GC → AT (Transition)	NED 2 years

evidence of mutation.

Details of mutations, mutation events and disease-free survival were reported in Table 8. The overall survival based on appropriate treatment during our study from 1994 to 1996 showed 6/40 = 15 per cent. When p53 mutations were carefully evaluated in the light of patients who survived their disease, it was found that all of the mutations except one were of transition type. As can be seen, the patients with stage I and II were expected to survive their disease at a higher rate than patients with stage III.

Discussion

p53 Overexpression and mutation

Our data showed overexpression of p53 protein in ovarian cancer at the level of 95 per cent (38/40) of all cases studied, while in most of the previously reported data, the rate was 60 per cent or less. This may be due to two main reasons. First, we used western transfer method to detect p53 protein which may be more sensitive than the most frequently used technique of immunohistochemical method. Second, our data had more late stage (36 of 40 stage III) and fewer early stage I and II disease (4 of 40 stage I and II). However, overexpression of p53 protein was seen in all of our stage I and II as well as stage III disease. Another interesting feature, was our ability to demonstrate the presence of p53 mutations by DNA sequencing in 31 samples out of 40 (78%), regardless of the status of their p53 protein. Most of the previous reports have their DNA sequenced if and when the samples were positive immunohistochemically for p53 protein or positive for SSCP[34] and therefore they could identify the presence of p53 mutationin about 50 per cent of the entire population of ovarian cancers. Our inability to identify mutations in 22 per cent of ovarian cancers that overexpressed p53 proteins raised the question of the overall sensitivity and specificity of p53 overexpression detected by any methods. As pointed out by other investigators[35,36] p53 overexpression may even occur in the absence of p53 gene mutation because of high-level cytoplasmic sequestration of wild-type p53 protein or mutation in the p53 promotor gene. The reverse is also true. Our data showed one case (MN 62) of point mutation at Codon 234 of Exon 7 in spite of negative overexpression of p53 protein (Table 3). This is not surprising, as one would expect that any one of these mutations, frameshift or deletion or nonsense mutations could stop the production of mutant p53 protein, resulting in negative Western Transfer Study.

The fact that when there is a lack of correlation between p53 protein overexpression and the presence of mutations it may not be possible to extrapolate the data of p53 protein overexpression to the presence or absence of p53 gene mutation. This point was also shown by Sjogren et al[37] in their study of p53 gene in 316 cases of breast cancers. In those that DNA sequencing was positive for p53 mutation, 33 per cent of them showed negative immunohistochemical reaction (false-negative IHC reactions). Meanwhile 30 per cent of positive immunohistochemical reaction cases failed to show any p53 mutations by direct DNA sequencing (false-positive IHC reaction). It would appear that if we were to capture most, if not all of the abnormal changes of the p53 suppressor gene or other genes, direct DNA sequencing may have to be included in all studies. Are there any built-in lack of sensitivity of DNA sequencing used at present? Most researchers would agree that the detection of mutation near the primer both forward and reverse may be problematic and that we may not be able to identify some mutations in or near the primers. This problem may be circumvented by using the primers to cover all coding regions from Exon 5 to 8, thereby eliminating some problems of detection of mutation in or near the primer of Exon 5, forward primer Exon 6, both forward and reverse primer of Exon 7.

Point mutation

Similar to the p53 mutations reported earlier in a variety of cancers including ovarian cancers,[9-18] the majority of p53 mutation from our data were missense mutations (Table 3) which result in amino acid substitutions in the p53 protein. These mutant p53 proteins have been shown to have many altered biochemical properties, including an increased half-life and ability to complex with p53 in order to inactivate wild-type p53 protein.[2,22,38] Our data have all but one missense mutation that contained microdeletion mutation causing frameshift at the end of the coding region of Exon 8 (Table 3). Comparing our data with those of Kohler et al,[22] Milner et al[24] and others[26-28] it was obvious that the p53 mutations did occur preferentially in highly conserved regions II,III (Exon 5), region IV (Exon 7) and region V (Exon 8). Two third

of p53 mutations from our data were in this highly conserved regions between Exon 5 and Exon 8 and none in Exons 1-3 and 9-11.[15,19-21,26-28] The other interesting features of our data was that there were 5 cases of ovarian cancers (MF79, EC59, MO73, MR63 and GC58) that have more than one mutation in their p53 gene (Table 3). These events did not appear to occur frequently in any studies, perhaps it was due to the tolerance limit of the p53 protein to survive and function. These interesting findings required further study to identify the rate of occurrence and the impact it will make to the protein and its function, or lack of. There has been sufficient evidence from many publications[19-24] including ours, indicating that p53 mutations occurred in all stages of ovarian cancer and perhaps in the borderline tumor as well.[38] This genetic event-p53 mutation - seemed to occur not only in the early stage but also in the late stage of carcinogenesis of all human malignancies.[10,15,20,24] p53 Mutation could perhaps be viewed as an initial step of carcinogenesis, which would require additional genetic events for the tumor to progress into an aggressive malignancy.

CpG mutations

It is believed by many investigators that CpG dinucleotides (cytosine-phospho-guanine) are preferentially involved in spontaneous mutations.[10,15,19,22-24] Since CGN is coded for Arginine (ARG), it is anticipated that this amino acid is likely to be a target for spontaneous mutation. The most likely process of mutation is deamination of the 5-methylcytosine that can change C to T or G to A transition. Our data showed CpG mutations at the rate of 15/41 (37%) in complete agreement with many reported data[12,15,24,28] including those of Kohler et al[22] 11/45 (24%). Since the spontaneous mutations were believed to play a major role in sporadic epithelial ovarian cancers, the mutation at CpG dinucleotide seems most likely to occur due to random errors in DNA synthesis preceding mitotic cell division.

Silent mutations

Point mutations occurring at one of the bases of the codon, but not resulting in the amino acid changes raised the possibility of genetic instability that may or may not lead to the initial step of carcinogenesis. The rate of silent mutations of 19.5 per cent in our study is large enough to warrant further study to understand the importance or lack of, in p53 suppressor gene mutation study.

p53 Mutations and survivorship

From Table 8, our data showed that details of mutation and mutation events in those who survived their cancer, even though the follow up was relatively short. However, the clinical and laboratory indicators clearly showed the freedom of cancer-free status. Two possible conclusions could be drawn from Table 8. The early stage cancer appeared to do well, as expected, and the other was that the survivors seemed to have the mutation event of Transition type.

Transition and transversion mutations

Our data showed a total of 41 mutations and a small deletion in 31 cases with 54 per cent of mutations to be Transition type (purine to purine or pyrimidine to pyrimidine) and the other 46 per cent of mutations were of Transversion type (purine to pyrimidine or pyrimidine to purine).[15,22] Analysis of the distribution of point mutations from our data (Tables 5 and 6) suggested a preferential mutation of the basepair G:C →A:T, occurring at the rate of 16/41 (39%). The least likely mutation was at G:C→T:A, occurring at the rate of 3/41 (7%). The high frequency of Transition mutations (G:C→A:T and A:T→G:C) in the p53 gene in our study is similar to those mutations observed in several other types of human cancers including colon and breast cancers.[9,10,12,15]

The occurrence of Transition mutations suggests that the mutation may arise from spontaneous errors in DNA synthesis and repair, an endogenous event that involves cellular proliferation rather than the exogenous event, implicating environmental factor.[15,22,24,27] The fact that Transversion type of mutations occurred in the ovarian cancers at the rate 46 per cent raises the probability that some other factors, not just spontaneous mutations, may be involved in DNA damage and repair of the p53 gene.

Summary

1. p53 Gene mutations were studied by two methods:

A) Mutant p53 protein study by immunoblotting (Western Transfer Method)

B) DNA sequencing of p53 gene

2. Results showed 95 per cent (38/40) overexpression of p53 protein and (31/40) 78 per cent of mutations in their p53 gene. Even though the correlation between p53 protein overexpression and the presence of mutation was not perfect, the data clearly showed that p53 mutation was found in most ovarian cancer, occurring in stage I, II and III.

3. Mutation of p53 occurred most frequently in Exon 8 (40%) in our study.

4. CpG dinucleotide mutations occurred at the rate of 15/41 = 37 per cent is in agreement with many other reports. CpG mutations are believed to be involved in spontaneous mutations.

5. Our data suggested that 50 per cent of p53 mutations in ovarian cancers may arise from spontaneous errors in DNA synthesis and repair (Transition Mutation) and the other 50 per cent may be involved in interaction of carcinogens with DNA.

6. Those who survived the cancer with appropriate treatment tend to have transition type of mutation 5/6 = 83 per cent.

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