CHAPTER - II

NARCOTICS USE &
p53 MUTATION
2.1 Background and Objective

2.1.1 Transcriptional regulation by p53

The transcription factor, p53, is the most importantly known tumor suppressor gene, and its functional inactivation is frequently associated with loss of genomic stability (Talos and Moll, 2010). It was originally thought to be an oncogene due to its binding to the SV40 T-antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), later it turned out to be a very potent apoptosis inducer and inhibitor of cell-cycle progression (Vogelstein et al., 2000). p53 knockout mice are susceptible to different tumor types (Donehower et al., 1992) and restoration of p53-function leads to the regression of tumors in vivo (Ventura et al., 2007; Xue et al., 2007).

The tumor suppressive function of p53 is mainly attributed to its ability to regulate numerous target genes at the transcriptional level. p53 can act both as a transactivator and as a repressor. The mechanism of transcriptional induction of p53 is well characterized. It involves DNA-binding to the p53 consensus site (El-Deiry et al., 1992). Transactivation is achieved by interactions with components of the general transcription factor TFIID like the TATA box binding protein (TBP) (Farmer et al., 1996; Liu et al., 1993) and TAFII31 (Lu and Levine, 1995). Numerous target genes upregulated by p53 have been characterized so far. Well-known targets are involved in apoptosis like Bax (Miyashita and Reed, 1995) and in cell-cycle control like the cyclin dependent kinase (CDK) inhibitor p21CIP1 (El-Deiry et al., 1993) or the inhibitor of Cyclin B/Cdc2 nuclear import, 14-3-3σ (Hermeking et al., 1997). For all known upregulated target genes DNA-binding of p53 is essential for regulation of gene transcription.

In addition to transcriptional induction, p53 has been shown to repress various target genes, but obviously by different mechanisms (Ho and Banchimol, 2003). Many of them are involved in cell-cycle control (Figure 11) and contribute to p53-induced cell-cycle arrest. Gene expression analyses showed that p53-dependent repression of target genes is associated with apoptosis after hypoxia treatment.
whereas transactivated genes were clearly underrepresented (Koumenis et al., 2001). Therefore, gene repression by p53 contributes to its tumor suppressive activity.

In principle, two categories of p53-repressed target genes can be classified: (I) genes that are regulated by direct interaction of p53 with target gene promoters or bound cofactors, (II) genes that are indirectly regulated by other p53 target genes. But most (if not all) of the mechanisms of transcriptional repression by p53 require its intact DNA-binding domain. Whether the DNA-binding domain is always needed for direct DNA binding to target gene promoters or interaction of p53 with other proteins through this domain can evoke p53-dependent repression, remains to be elucidated.

**Figure 11: The tumour suppressor protein p53 can interfere in multiple ways with cell-cycle progression (Bohlig and Rother, 2011)**
All of the cell-cycle regulators shown in this figure are transcriptionally downregulated by p53 (black lines with horizontal bars). Inhibition (dotted lines) and activation (dotted arrows) of a subset of regulation between different p53 target genes is indicated.

2.1.2 p53 and cancer

p53 mutations are found in approximately 50% of human cancers (Soussi et al., 2001). Apart from the fact that tumor cells must select for inactivation of the p53 network that safeguards the cell from various types of insults, these mutations are oncogenic and have been the subject of extensive studies providing a better understanding of their origin (Greenblatt et al., 1994).

The unique feature of mutated p53 compared to other tumor suppressor genes is its mode of inactivation. While most tumor suppressor genes are inactivated by mutations leading to absence of protein synthesis (or production of a truncated product), but more than 80% of p53 alterations are missense mutations that lead to the synthesis of a stable full-length protein (Soussi et al., 2001). This selection to maintain mutant p53 in tumor cells is believed to be required for both a dominant negative activity to inhibit wt p53 expressed by the remaining allele, and for a gain of function that transforms mutant p53 into a dominant oncogene (Figure 12) (Soussi and Beroud, 2003; Lane and Benchimol, 1990; Dittmer et al., 1993).

Figure 12: p53 and cancer
In the classical situation (left, mut I), the first hit leads to inactivation of p53 without affecting the activity of the second allele. However, this loss of function may have certain consequences for the cell. The second hit leads to complete loss of function of p53. In the case of a dominant negative mutant (middle, mut II), an estimated 1/16 of the tetramers theoretically have 4 wild-type monomers if the two proteins are expressed in identical quantities and tetramerization is not affected by the mutation. If the dominant negative effect of a single monomer is sufficient to inactivate p53, then the remaining activity will be 6.25%. In the case of partial penetrance of the dominant negative effect, the activity gradient will be between 6% and 50%. Loss of the second allele may not be mandatory, depending on the remaining activity. In the case of a mutant with a gain of function (right, mut III), the situation is more complex with an important combinatorial effect. Loss of the second allele may also be unnecessary in this case. This particular situation of the p53 gene results from: (i) its particular mode of inactivation by missense mutations and (ii) its tetrameric structure.

An important feature of the p53 protein is the extreme flexibility and fragility of the DNA binding domain (residues 90–300) (Milner, 1995), as more than 200 of the 393 residues have been found to be modified and several residues have sustained multiple alterations. Most p53 mutations are localized in the DNA binding domain of the protein (residues 100–300) leading to a bias of p53 mutation analysis, as more than 80% of p53 mutation studies focus on exons 5–8 (residues 126–306) (Figure 13) (Soussi et al., 2001).

Figure 13: Structure of p53 and the hotspots of mutations
During the process of carcinogenesis, \( p53 \) mutations mostly arise sporadically in one allele, resulting in cells expressing both wild-type and mut\( p53 \), where the latter might suppress the tumor suppressor activities of the former by oligomerizing with it through the C-terminal tetramerization domain (Sigal and Rotter, 2000). Eventually, in the course of tumor progression, the remaining wt \( p53 \) allele is often lost (mostly by deletion), further enhancing tumorigenesis.

### 2.1.3 Relation between \( p53 \) and tobacco smoke

It is widely stated that G→A transitions at CpG sites in the \( p53 \) gene result from deamination of 5-methylC, is a plausible result of endogenous processes. Indeed, a recent study (Chen et al., 1998) demonstrates that cytosine methylation greatly enhances guanine alkylation at all CpG sites in the \( p53 \) gene by a variety of carcinogens. \( O^6 \)-Alkylguanines, such as those formed from nitrosamines, are another likely cause of G→A transitions (Singer and Essigmann, 1991). With respect to the origin of G→T transversions in the \( p53 \) gene, a previous study (Denissenko et al., 1996) demonstrated that the carcinogens selectively forms adducts at CpG sites in codons 157, 248, and 273 three major sites of mutation in the \( p53 \) gene. However, studies (Chen et al., 1998, Denissenko et al., 1997) have also clearly shown that methylated CpG sites are targets of a variety of activated carcinogens. Thus, it strongly suggests that other DNA-reactive compounds and DNA adducts derived from tobacco smoke would have similar effects.

### 2.1.4 Objective

From the prevalence and risk factor analysis data of chapter I, we have shown that the use of narcotics [3.429 (0.54, 21.66)] as a risk for the development of ovarian cancer. In this regard we ought to describe the relationship between \( p53 \) mutation and status of narcotics use.
2.2 Materials and Methods

2.2.1 Study subjects and sample collection
The study subjects were as per the section 1.3.3. Blood samples were collected in EDTA containers and transported to the laboratory on ice. The samples were stored at -20°C until use.

2.2.2 DNA extraction
The DNA from the blood samples were extracted as per the procedure described earlier (Mullenbach et al., 1989) with slight modifications. 200 µl of blood cells were mixed with 500 µl of nucleus lysis buffer (10mM Tris HCl; 400 mM NaCl; 2 mM Na₂EDTA pH 8.2), 50 µl of 10% SDS and 10 µl of proteinase K (10 mg/ml). The mixture was incubated at 55°C for 3 hours. To the lysate 100 µl of 5M NaCl was added and extracted with phenol: chloroform: isoamylalcohol (25:24:1). The DNA was precipitated from the aqueous phase with twice the volume of ethanol. The precipitated DNA was washed with 70% ethanol and air dried at room temperature. The DNA pellet thus obtained was resuspended in 25 µl of TE buffer and stored at -20°C until use. The quality of the DNA was determined by measuring the absorbance at 260 and 280 nm and visualized it on 0.8% agarose gel stained with ethidium bromide.

2.2.3 Polymerase chain reaction
The oligonucleotide primers and the length of the amplicons for exons 5 through 9 are tabulated in table 3.

Table 3: The oligonucleotide primers used for amplification of p53

<table>
<thead>
<tr>
<th>Exon number</th>
<th>Primer name/sequence</th>
<th>Amplicon (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 and 6</td>
<td>Ex5F/ 5’TTCTCTTTCTGCAGTACTC3’ Ex6R/ 5’AGTTGCAAACCAGACCTCAG3’</td>
<td>408</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>PD7/5’CAAGTGCTCCTGACCTG GAGTC3’ PU7/5’GTGTGGTCTCCTGGTTGCTTG3’</td>
<td>139</td>
<td>55</td>
</tr>
<tr>
<td>8 and 9</td>
<td>Ex8F/ 5’CCTATCCCTG AGTAGTGGTAA3’ PD9/5’CCAAGACTTAGTACCTGAACCGGTG3’</td>
<td>332</td>
<td>55</td>
</tr>
</tbody>
</table>
PCRs were carried out in 25 µl reaction volume containing 50–100 ng blood DNA, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM of each primer and 1 unit of Taq DNA polymerase (Fermentas, Germany). The target DNA was denatured at 94°C for 4 min, where after, 30 cycles of amplification were performed with the thermocycler (Eppendorff, Germany) under following conditions: DNA denaturation at 95°C for 30 s, primer-annealing at 60°C for 30 s and primer-extension at 72°C for 1 min. For all reactions, the final extension for 10 min at 72°C was provided. 5 µl of the amplified product was run on an agarose gel stained with ethidium bromide and visualised in gel documentation system (BioRad, USA).

2.2.4 SSCP-analysis (Single-Stranded Conformation Polymorphism)
One microliter of the PCR product was diluted with 10 volumes of denaturing solution containing 95% formamide, 20 mM EDTA (pH 8.0), 0.05% xylene cyanol and 0.05% bromphenol blue, heat-denatured for 10 min at 99°C and chilled on ice for 10 min. Mutations in DNA was demonstrated by running case patients and control subjects PCR products in the adjacent lanes, on a 12% non denaturing acrylamide gel containing 5% glycerol using 1xTAE buffer (Spinardi et al., 1999). The fragments were visualized after silver staining.

2.2.5 Direct sequencing
DNA segments identified as having altered mobility by SSCP were evaluated by DNA sequence analysis with ABI Prism BigDye Terminator cycle sequencing at Macrogen (South Korea). Bio-Edit software was used for analyzing the variations in the sequences of the study population on comparison with the reference sequence NC_000017 version 19 from Genbank.

2.2.6 Statistical analysis
The data were coded and analyzed using the SPSS 17.0 statistical software. The odds ratio and the corresponding 95% confidence intervals were calculated by unconditional logistic regression and maximum likelihood estimation. Tests of statistical significance were based on difference in the log likelihoods, and all P values are 2-sided.
2.3 Results and Discussion

2.3.1 SSCP analysis

All the 72 ovarian carcinoma cases and the control subjects were analyzed for p53 exons 5 to 9 by SSCP to demonstrate conformational band shifts (Figure 14-16). The samples that scored band shifts were subjected for direct sequencing.

*Figure 14: SSCP analysis to determine conformational band shifts for exon 5 and 6*  
*Figure 14 a :*
Figure 14b:

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23: Case samples (CAO29 to 36, 44, 45, 66); Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24: Control samples (CON 27, 30, 31, 33, 37, 38, 40, 43, 44, 45, 47, 48)

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23: Case samples (CAO68, 70, 71, 37, 39, 40, 41, 42, 43, 59, 60, 38, 48, 43); Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22: Control samples (CON 51, 55, 57, 28, 29, 60, 62, 109, 110, 111)

Figure 14c:

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17: Control samples (CON58 to CON62 CON23 to CON26); Lane 2, 4, 6, 8, 10, 12, 14, 16: Case samples (CAO30 to CAO33; CAO25 to CAO28)

Lane 1 to 17: CON61, 63, 64 to 79

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Figure 15: SSCP analysis to determine conformational band shifts for exon 7

Figure 15a:

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27: Control samples (CON 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16)
Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26: Case samples (CAO1, 2, 5, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18)

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27: Control samples (CON 71, 72, 73, 74, 75, 76, 81, 82, 83, 84, 85, 86, 87, 88)
Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26: Case samples (CAO19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 30, 31, 32)

Figure 15b:

Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27: Control samples (CON 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101)
Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26: Case samples (CAO34, 35, 45, 46, 48, 49, 50, 51, 52, 53, 55, 56, 57)

Lane 1, 3, 5: Control samples (CON 102, 103, 104)
Lane 2, 4, 6: Case samples (CAO58, 59, 60)
Figure 16: SSCP analysis to determine conformational band shifts for exon 8 and 9

Figure 16a:

Lane 1, 3, 5, 7, 9, 10, 12, 14, 16, 18, 19, 21, 23, 25, 27: Case samples (CAO1, 2, 5, 7, 8, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21); Lane 2, 4, 6, 8, 11, 13, 15, 17, 20, 22, 24, 26: Control samples (CON 17, 72, 73, 74, 75, 76, 77, 78, 53, 54, 55, 57)

Figure 16b:

Lane 1, 3, 5, 7, 9, 10, 12, 14, 16, 18, 19, 21, 23, 25, 27: Case samples (CAO23, 24, 26, 15, 22, 27, 28, 29, 30, 31, 32); Lane 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 22, 24, 26: Control samples (CON 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 22, 23, 24, 25)

Lane 1, 3, 5, 7, 9, 10, 12, 14, 16, 18, 19, 21, 23, 25, 27: Case samples (CAO 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52); Lane 2, 4, 6, 8, 11, 13, 15, 17, 20, 22, 24, 26: Control samples (CON 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 44, 45)

Lane 1, 4, 7, 10, 13, 16, 19, 22, 25: Case samples (CAO54, 56, 57, 53, 58, 59, 60, 61, 63) Lane 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 21, 23, 24, 26, 27: Control samples (CON 46, 47, 48, 49, 50, 51, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 105)
2.3.2 p53 variations

Of all the 72 ovarian carcinoma cases analyzed in this study, 18 (25%) were found to contain one or more of p53 variations. One of the variations was found to be polymorphism because the changes were silent predicting no alteration in the aminoacid sequence and moreover the variation was observed in 5 of the control cases analysed, leaving 17 alterations (17/72; 23.6%) detected in ovarian carcinoma. Data on the nature of individual p53 variations are presented in table 4 and figure 17. Majority of the mutations (9/17) were frameshift, 7 deletions and 2 were insertions. One case patient was found to have two alterations one in the codon 246 and another in 270. Other than the single polymorphism observed among 5 control cases no other p53 variations were detected among the control subjects.

Table 4: Summary of mutation analysis of p53

<table>
<thead>
<tr>
<th>Cases</th>
<th>Histology</th>
<th>Exons</th>
<th>Codons</th>
<th>Nucleotide</th>
<th>Aminoacids</th>
<th>Mutation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Adenocarcinoma</td>
<td>5</td>
<td>136</td>
<td>CAA→-AA</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td>11</td>
<td>Serous</td>
<td>5</td>
<td>139</td>
<td>AAG→A-G</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td>27</td>
<td>Serous</td>
<td>5</td>
<td>139</td>
<td>AAG→A-G</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td>33</td>
<td>Endometrioid</td>
<td>5</td>
<td>136</td>
<td>CAA→CA-</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td>38</td>
<td>Serous</td>
<td>5</td>
<td>138</td>
<td>GCC→GGC</td>
<td>Ala→Gly</td>
<td>Missense</td>
</tr>
<tr>
<td>53</td>
<td>Serous</td>
<td>5</td>
<td>139</td>
<td>AAG→CAG</td>
<td>Lys→Gln</td>
<td>Missense</td>
</tr>
<tr>
<td>50</td>
<td>Serous</td>
<td>5</td>
<td>140</td>
<td>ACC→AACC</td>
<td>Frameshift</td>
<td>1-bp insertion</td>
</tr>
<tr>
<td>34</td>
<td>Serous</td>
<td>7</td>
<td>195</td>
<td>GAT→GAC</td>
<td>Ile→Val</td>
<td>Missense</td>
</tr>
<tr>
<td>35</td>
<td>Serous</td>
<td>7</td>
<td>246</td>
<td>CAT→C-T</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>270</td>
<td>TTT→TTG</td>
<td>Phe→Leu</td>
<td>Missense</td>
</tr>
<tr>
<td>17</td>
<td>Mucinous</td>
<td>8</td>
<td>268</td>
<td>AAC→-AC</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
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<tr>
<td>60</td>
<td>Serous</td>
<td>8</td>
<td>271</td>
<td>GAG→GAA</td>
<td>Glu→Glu</td>
<td>Missense</td>
</tr>
<tr>
<td>47</td>
<td>Serous</td>
<td>8</td>
<td>275</td>
<td>TGT→-GT</td>
<td>Frameshift</td>
<td>1-bp deletion</td>
</tr>
<tr>
<td>36</td>
<td>Serous</td>
<td>8</td>
<td>280</td>
<td>-AGA→GAGA</td>
<td>Frameshift</td>
<td>1-bp insertion</td>
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<tr>
<td>57</td>
<td>Unclassified</td>
<td>8</td>
<td>266</td>
<td>GGA→GCA</td>
<td>Gly→Ala</td>
<td>Missense</td>
</tr>
</tbody>
</table>
Figure 17: Summary of mutations analysed in $p53$

Figure 17a

<table>
<thead>
<tr>
<th>Reference</th>
<th>Case</th>
<th>Codon</th>
<th>Mutation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCCTCTCGGAGTGGTTTC</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>GGCAGCTG</td>
<td>AAGCTAGGAAGTTTTCGACCT</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGGGTTTGAT</td>
<td>TCCACACACACAAACACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 17b

<table>
<thead>
<tr>
<th>Reference</th>
<th>Case</th>
<th>Codon</th>
<th>Mutation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGGGACAAATGTTTTTCGACATCTGGGCAG</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>GGCAGAACC</td>
<td>TGGGTTTTGACCTTCCACACACACAAACACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGGGTTTGAT</td>
<td>TCCACACACACAAACACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Figure 17c

**Codon 246 DelA**

```
TAGTACGCGGGGGAGACCTCCTGTCGCCTGC
CCATGCAGCAACTGTACCATGATGGTACATGAG
GGTACAGTCAGACCAAGAGAGAACAAAC
```

Figure 17d

**Codon 195 (translocation)**

```
GCGGGGATGACAGCTGCATATGTATAG
GTOGGTCGGGGTTGCATGCGCCACCAT
GCAAGGAACTGGATCACATGAGTG
GTATGGTGGGTGATGATGATCGAG
CCAACCTAGGAGACCAAC
```

**Codon 330 (transversion)**

```
ACCATGGCGTACAGCTTTGAAGT
GCGGATGTGGCGCTGTGAGGAGAAG
ACGGCCGACAGGAAAGAAGAATCTC
CAAGAAAGGGAGACCTCACCCAGAG
CTGGCACCAGGGAGCAACTAAAGCGAG
TAAGCAAGCGAGACAGAAGAAGGCGTG
GAAGGAGCAACAGGTGGATGATGCC
TCAAGATTCACCTTATACACCTGCTT
```

**Codon 268 (delA)**

```
AACAGCT
```

**Codon 271 (InS)**

```
TTTGATG
```

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Figure 17e

Figure 17f

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The p53 is a metal binding transcription factor. The normal condition of ovarian epithelium is to have low or undetectable steady state levels of p53 proteins while cancers containing high levels are aberrantly expressing this oncogene. Generally mutations render p53 oncogenic and also increase the half life of this normally unstable protein. It is widely held that most cancers are the result of multiple independent genetic lesions and that therefore it is likely that p53 acts in concert with other oncogenes in the ovary during tumorigenesis. p53 mutations do not occur in a random fashion along the coding sequence but are typically clustered at so called mutation hotspots. All these hotspots are within the DNA binding domain of p53 protein spanning approximately 180 amino acids from codon 120 to 300. Certain studies have shown that mutation of the p53 gene is in itself is probably insufficient to induce epithelial ovarian malignancies (Marks et al., 1991). However p53 mutation is common among ovarian cancers and must have been selected for and maintained during tumour progression.

In the present study we reported 25% of the investigated ovarian carcinoma cases to contain one or more p53 variations of those the majority being single nucleotide
mutations. The observed frequencies (25%) in this study are in the lower end, but comparable to those reported in the literature (30% to 80%) (Aunoble et al., 2000; Wertheim et al., 1994; Kohler et al., 1993; Milner et al., 1993; Shahi et al., 2000; Reles et al., 2001; Wen et al., 1999; Havrilesky et al., 1998; Okuda et al., 2003). Of the 17 mutations found, only 8 have previously been reported. Out of 8, 5 in ovarian carcinoma and 3 have been reported in other cancer types. Nine mutations have been reported for the first time and not available in the p53 database. The differences in frequencies between the present study and frequency reported elsewhere may be due to population based differences in the molecular epidemiology of p53 mutation.

2.3.3 Functional analysis of the p53 variants

Different p53 mutants can display different degrees of impairment of their transactivation potential, reflecting the nonidentical impact of individual mutations on the overall structure of the mutant protein (Pan and Haines, 2000; Inga et al., 2002; Kato et al., 2003). The variations in the transcriptional regulation by mutant p53 were determined from the available data from the IARC p53 database. Out of the 17 mutations reported in the present study, 8 have been previously reported and its variation in transcriptional regulation is given in table 5. All the mutations were found to be deleterious as the predominantly mutated domain was found to have DNA binding. The transactivating potential of p53 for RGC, WAF1, Bax, PIG3, JNK and PCNA are lost due to 140InsA, A139C, A195G, 246delA, G271A, T270G, T274G mutations (Figure 18). The mutation C138G, A139C, G271A caused a loss of function of upregulation of P21, WAF1, MMD2 and downregulation of bcl2 (Table 5). As a result of these three mutations two gain of function has been reported, one being the upregulation of MRP1 (Multi-drug resistant protein-1) and other being CDDP resistance.

In this regard it can be reported that these 3 mutations are supposed to cause drug resistance of the tumour making it non-responsive to chemotherapeutic drugs and hence decreased survival rates, increased progressive type of tumour, increased remission and increased relapse condition.
**Table 5: Summary of functional analysis of p53 mutations**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Exon</th>
<th>Codon</th>
<th>Domain/Residue function</th>
<th>AGVGD/SIFT score</th>
<th>Lost function</th>
<th>Gained function</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>5</td>
<td>138</td>
<td>DNA binding/partially exposed</td>
<td>Deleterious/deleterious</td>
<td>Up regulation of P21Waf1 Down regulation of bcl2 Up regulation of MMD2</td>
<td>Up regulation of MRP1</td>
</tr>
<tr>
<td>M2</td>
<td>5</td>
<td>140</td>
<td>DNA binding/partially exposed</td>
<td>Deleterious/deleterious</td>
<td>Transactivation of RGC partial</td>
<td>ND</td>
</tr>
<tr>
<td>M3</td>
<td>5</td>
<td>139</td>
<td>DNA binding/partially exposed</td>
<td>Deleterious/deleterious</td>
<td>Transactivation of RGC, WAF1, Bax, PIG3</td>
<td>Upregulation of MRP1</td>
</tr>
<tr>
<td>M4</td>
<td>7</td>
<td>195</td>
<td>DNA binding/buried</td>
<td>Deleterious/neutral</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M5</td>
<td>7</td>
<td>246</td>
<td>DNA binding/buried</td>
<td>Deleterious/deleterious</td>
<td>TA (RGC, BAX, PIG3, PCNA)</td>
<td>ND</td>
</tr>
<tr>
<td>M6</td>
<td>8</td>
<td>271</td>
<td>DNA binding/buried</td>
<td>Deleterious/deleterious</td>
<td>TA (RGC), JNk and MDM2 binding</td>
<td>CDDP resistance</td>
</tr>
<tr>
<td>M7</td>
<td>8</td>
<td>270</td>
<td>DNA binding/buried</td>
<td>Deleterious/deleterious</td>
<td>TA (RGC, PCNA)</td>
<td>ND</td>
</tr>
<tr>
<td>M8</td>
<td>8</td>
<td>274</td>
<td>DNA binding/buried</td>
<td>Deleterious/deleterious</td>
<td>TA (RGC, WAF1, BAX, PIG3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND- Not determined

M1- C138G; M2-140InsA; M3 – A139C; M4 – A195G; M5 – 246delA; M6 – G271A; M7 – T270G; M8- T274G
2.3.4 Authentication for gained function

2.3.4.1 Upregulation of MRP-1

Standard treatment for epithelial ovarian carcinomas is cytoreductive surgery followed by chemotherapy. This therapy results in variable efficacy rates with frequent recurrences even at early stages. Moreover, the tumours become eventually resistant to chemotherapy. These clinical data indicate that long-term prognosis in ovarian carcinoma depends on intrinsic and acquired resistance to chemotherapy. Studies of cell culture systems have established that tumour cells exposed to only a single drug expressed cross-resistance to a broad range of structurally and functionally dissimilar drugs. This phenomenon is called MDR. While various mechanisms may contribute to MDR, the primary ones are the so-called “pump” and “non-pump” forms of resistance (Pakunlu et al., 2003; Minko et al., 2004). The basic mechanism of non-pump resistance is activation of cellular antiapoptotic defence. Pump resistance or transport-mediated resistance is due to the decreased concentration of the active drug in target cells because of decreased drug uptake or
increased drug efflux across tumour cell membranes, or to increased drug efflux through cytoplasmic vesicles.

Certain proteins are able to transport toxic materials and drugs across cellular membranes, against a concentration gradient, decreasing their intracellular concentration (Stein, 1967). A large subclass of these proteins is ATP-binding cassette (ABC) proteins, which are expressed in both normal and malignant cells. The main ABC transporters are P-gp and MRP1. Such proteins act as energy-dependent efflux pumps capable of expelling a large range of xenobiotics, including doxorubicin and other cytotoxic drugs derived from natural products, out of the cell.

MRP1 is a 190 kDa multidrug resistance protein which is probably involved in an antioxidant defence mechanism. MRP1 can act as a transporter of glutathione conjugates (Muller et al., 1994). Although the precise role of the glutathione detoxification pathway in the MDR phenomenon has not yet been fully elucidated, the isoenzymes of the glutathione-S-transferase (GSTs), namely the subclass GSTpi (EC 2.5.1.18), have been extensively reported to be overexpressed in tumour cells displaying the MDR phenotype (Keith et al., 1990; Buser et al., 1997; Ferrandina et al., 1997; Frassoldati et al., 1997; Silvestrini et al., 1997; Boku et al., 1998; Stoehlmacher et al., 2002; Oudard et al., 2002; Cullen et al., 2003; Galimberti et al., 2003; Bennaceur-Griscelli et al., 2004). However, the role of iGSTs proteins remains controversial in the literature.

Overexpression of MRP1 has been detected in a variety of tumor types, and has also been associated with drug resistance or poor patient outcome in lung cancer (Ota et al., 1995), breast cancer (Nooter et al., 1997) and gastric cancer (Endo et al., 1996). Previous studies have also demonstrated expression of MRP1 in ovarian carcinomas (Izquierdo et al., 1995; Kavallaris et al., 1996) but neither of the studies showed a relationship with expression and response to chemotherapy or progression-free survival.

2.3.4.2 MRP-1 overexpression studies-IHC analysis

The Patients recruited in the study were treated with cytoreductive surgery and staged according to the International FIGO classification. All patients with ovarian
carcinoma staged III–IV were treated with chemotherapy containing carboplatin and/or paclitaxel (a multidrug related regimen). The response to first-line chemotherapy was evaluated by clinical or radiological examination according to Gynecologic Oncology Group criteria. In patients with 50% reduction of tumour was considered a partial response, and complete disappearance of the disease either by clinical or radiological examination was considered a complete response. Stable disease was a steady state of response either less than a partial response or progression. Progressive disease was defined as an increase of at least 25% or the appearance of any significant new lesions. Patients with incomplete response to first-line chemotherapy or recurrent tumors were treated with a variety of second-line chemotherapeutic protocols. Thirty nine formalin fixed, paraffin embedded ovarian carcinoma tissues were retrieved from the archives of Department of Pathology, Dr.GVN Institute of Oncology. The histological diagnosis was made on haematoxylin-eosin stained paraffin sections and they were classified according to WHO classification.

Immunohistochemistry using representative formalin fixed paraffin embedded tissue blocks was carried out. The sections were deparaffinised followed by antigen retrieval (autoclave retrieval at 121°C for 10 mins in 10mM sodium citrate buffer, pH 6.0). A monoclonal antibody to MRP1 (kind gift from Dr. Cole of Queensland University) was diluted in 1:500 in blocking solution and reacted overnight at 4°C. An anti-mouse IgG HRP detection system with diaminobenzidine tetrachloride (Sigma Aldrich, USA) was used. The sections were counter stained with hematoxylin and embedded. The results of immunohistochemistry were interpreted as strongly positive (+3) only when >10% of the tumour cells demonstrated a strong complete membrane staining; as weakly positive (+2) when weak to moderate complete membrane staining was observed in >10% of the tumour cells; and as negative (0/+1) when a barely perceptible or no membranous staining was observed.

During the study period, 80 consecutive patients with primary epithelial ovarian carcinoma were included. 8 cases were excluded from the study as they were unavailable for follow up. Seventeen patients with stage I or stage II carcinoma did
Genetic, molecular and microbiological basis for ovarian carcinoma from a low incidence population

S.Shanmughapriya, Department of Biochemistry, Bharathidasan University

not receive chemotherapy. Sixteen cases were excluded from the study because of insufficient frozen material. Therefore, tissue blocks from 39 patients treated with chemotherapy were included in the study.

Nine patients had a complete clinical response (23.1%), 3 (7.6%) had a stable disease, 18 (46.2%) had a progressive disease, 6 (14.5%) had relapsed disease, 3 (7.6%) died of the disease during or after first line chemotherapy.

MRP1 protein was strongly expressed in 17 tumour samples (43.6%). The staining pattern was both strong diffuse cytoplasmic as well as membraneous in >10% of tumour cells. Staining intensity varies from low to moderate. In some tumours with strongest MRP1 staining, sporadic membranous staining was observed (Figure 19).

2.3.4.3 Relation between immunostaining and p53 mutation

No statistically significant relationship could be established between the p53 mutation and immunostaining for MRP1 (Table 6). All the ovarian carcinoma cases found to be mutated for p53 showed an upregulation of MRP-1 expression. 55.6% of the cases with wild type p53 also showed an upregulation of MRP-1 expression. Thus the results of the present study envisaged that the MRP-1 upregulation not only depends on the mutation of its regulatory gene, p53, but instead probably on the chemotherapy regimen adopted for the first line therapy.

<table>
<thead>
<tr>
<th>P53 status</th>
<th>MRP1 expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>14 (82.4)</td>
<td>3 (17.6)</td>
</tr>
<tr>
<td>Wild type</td>
<td>14 (63.6)</td>
<td>8 (36.4)</td>
</tr>
</tbody>
</table>

Chi square test: mutated Vs wild type

Table 6: Relation between MRP-1 immunostaining and p53 mutation
Figure 19: Immunohistochemistry to study MRP-1 expression and its correlation with p53 mutation

[M1 positive, Wild type Tp53, Wild type P53 images]

[M3 positive, M3 positive, Wild type (TP53) images]

[M3 positive, M3 positive, Wild type (TP53) images]

[Wild type (TP53), Wild type (TP53), Wild type (TP53) images]

[Wild type P53, Wild type P53 images]
2.3.4.4 Relation between immunostaining and response to first-line chemotherapy

96.4% of the cases expressing MRP1 showed no response to the chemotherapy regimen employed and thus a statistically significant relationship could be established between response to first line chemotherapy and immunostaining for MRP1 (Table 7). All the patients included in the study were treated with regimens containing MDR related regimens and hence higher chance of poor therapy outcome has been predicted.

Table 7: Tumor response to first-line chemotherapy: patients treated with cisplatin in combination with MDR drugs

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Study subjects (N=39)</th>
<th>Treatment Regimen Response (%)</th>
<th>Non-Response</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1 Negative</td>
<td>21</td>
<td>8 (38.1)</td>
<td>13 (61.9)</td>
<td>0.0149*</td>
</tr>
<tr>
<td>MRP1 Positive</td>
<td>18</td>
<td>1 (5.6)</td>
<td>17 (94.4)</td>
<td></td>
</tr>
</tbody>
</table>

*- Chi-square test: Response Vs non-response

MRP1 is overexpressed in many MDR cell lines (Zaman et al., 1993; Barrand et al., 1994). In the present study, immunostaining for MRP1 was observed in 43.6% of ovarian carcinomas, whereas in a previous study by Izquierdo et al., 1995 in 68% of 57 stage III and IV ovarian carcinomas. Although elevated MRP1 mRNA levels have been reported in several human cancers, the role of MRP1 in clinical drug resistance is not fully defined (Nooter et al., 1995). The present study has shown a relation of MRP1 staining with response to chemotherapy. But previous reports have not reported such kind of relation with response to chemotherapy or survival (Iquierdo et al., 1996). Using RT-PCR, Kavallaris et al., 1996 detected moderate MRP1 expression in all ovarian carcinomas and high MRP1 expression in 43% of ovarian carcinomas without relation to progression-free survival. In vitro and clinical data thus far do not point to a relation for MRP1 with response to platinum- and/or paclitaxel-containing chemotherapy.

Our results confirm that the expression level of MRP1 strongly correlates with the degree of response to chemotherapy and that the levels of MRP1 expression usually increases in relapsed disease. Advances in diagnostic techniques and the possibility of accurate selection of patients with tumours that overexpress this transporter.
should result in more successful treatment of ovarian carcinoma with drugs that are not dependent on the ABC transport system, with new targeted therapies, or with additional ABC inhibitor therapies.

2.3.5 P53 mutations in relation to clinicopathological parameters

From the results of the epidemiological study from this particular population we have shown that the use of narcotics is a risk for the development of ovarian cancer. In this regard we ought to describe the relationship between p53 mutation and status of narcotics use. Surprisingly 25.9% of non-narcotic users carried p53 mutation on comparison with 22.2% of narcotics users. No significant differences were found between a p53 mutation and p53 wild type in regard to histological type of tumour (serous adenocarcinoma, mucinous adenocarcinoma, endometrioid adenocarcinoma and others) and a history of narcotics usage (P = 0.438) (Table 8).

**Table 8: Correlation between P53 mutation and histopathology, history of narcotics use**

<table>
<thead>
<tr>
<th>Variables</th>
<th>P53 mutation status (N = 72)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P53 mutated No (%)</td>
<td>P53 wild type No (%)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>14 (27.5)</td>
<td>37 (72.5)</td>
<td>1.608 (0.46, 5.62)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
<td>1.529 (0.13, 17.94)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>0.735 (0.08, 7.04)</td>
</tr>
<tr>
<td>Other types</td>
<td>2 (15.4)</td>
<td>11 (84.6)</td>
<td>0.489 (0.09, 2.45)</td>
</tr>
<tr>
<td>Narcotics use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (22.2)</td>
<td>14 (77.8)</td>
<td>0.585 (0.15, 2.27)</td>
</tr>
<tr>
<td>No</td>
<td>14 (25.9)</td>
<td>40 (74.1)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

From the data, it shows that the narcotics users are at a reduced risk of acquiring P53 mutation compared to women without a history of narcotics use. Codons 157 and 158 are considered as hotspots specific for lung cancers of smokers and G-T transversions at this codon is the most common substitution. In the present study neither of the codon nor G-T transversions has been reported among p53 mutation carriers with a history of narcotics use. This evidenced that p53 mutation in this study population is not strongly related to tobacco smoke. On the other hand it can also be concluded that different tobacco smoke compounds may exert carcinogenic,
co-carcinogenic, or tumor promoting effects in an organ- and tissue specific manner, depending on the rate of accumulation and metabolism at various sites in the body. Hence our hypothesis of correlating narcotics usage and p53 mutation does not hold good. Thus it can be concluded that narcotics usage is not a major factor for p53 mutation in this type of carcinoma.