**WAF1/CIP1** structural abnormalities do not contribute to cell cycle deregulation in ovarian cancer

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**Summary** Mutations in the **WAF1/CIP1** gene were not found in 36 ovarian carcinomas, including tumours with loss of heterozygosity at the **WAF1/CIP1** locus and/or lacking p53 mutations. In addition, no association was demonstrable between a polymorphism in a conserved region of the **WAF1/CIP1** gene and ovarian carcinoma

**Keywords:** **WAF1/CIP1**; P21; P53; chromosome 6; ovarian carcinoma

The p21 protein is a cyclin-dependent kinase (CDK) inhibitor encoded by the **WAF1/CIP1** gene (Harper et al., 1993; Xiong et al., 1993; El-Deiry et al., 1993). It is a mediator of p53 (El-Deiry et al., 1993, 1994), a tumour-suppressor protein mutated in a wide variety of tumours (Hollstein et al., 1991). It provides a link between cell cycle regulation and p53 expression, as its induction by p53 results in inhibition of cyclin/CDK-mediated phosphorylation of retinoblastoma (RB) protein. The latter is required for G1 to S transition (Harper et al., 1993; El-Deiry et al., 1993). **WAF1/CIP1** may have additional roles in cell cycle regulation and cancer because it can also be induced by a p53-independent pathway (Michieli et al., 1994) and is able to inhibit DNA replication and repair independently of the CDKs (Waga et al., 1994; Li et al., 1994).

It was proposed that mutations in the **WAF1/CIP1** gene may contribute to the development of ovarian tumours lacking p53 mutations (El-Deiry et al., 1993), as these tumours frequently show losses of heterozygosity in a region of chromosome 6p that includes the **WAF1/CIP1** locus (Cliby et al., 1993; Foulkes et al., 1993; Wan et al., 1994). A tumour-suppressor role for p21 is further supported by the fact that its expression inhibits the growth of cultured tumour cells (El-Deiry et al., 1993). The finding of point mutations in ovarian tumours with loss of heterozygosity involving the **WAF1/CIP1** locus, especially in the absence of p53 mutations, would strongly support a role for this gene as a tumour suppressor important for the control of ovarian tumorigenesis. We therefore sequenced the **WAF1/CIP1** gene in ovarian tumours already characterised for the presence or absence of loss of heterozygosity at this locus and for p53 mutations.

**Materials and methods**

**Source and handling of tissue and blood samples**

All tumours were of ovarian epithelial origin. The source and handling procedures were described previously (Wan et al., 1994). DNA from normal individuals was extracted from white blood cells obtained from volunteers of mixed ethnic origins but with a predominance of those of Hispanic origin.

**Mutational analysis of WAF1/CIP1 gene**

The entire exon 2 and the 5’ portion of exon 3 of the **WAF1/CIP1** gene were enzymatically amplified from genomic DNA using intron-based primers (obtained from Dr Bert Vogelstein, Baltimore, MD, USA). The sense and antisense primers used for amplification of exon 2 were 5’-TGGAAGAGTAGGAGAGA-3’ and 5’-ATCTCTGTTCCCTATCAAGT-3’ respectively. Sense and antisense primers for amplification of the 5’ portion of exon 3 were 5’-GCGAGCTCCGCGCCG-3’ and 5’-CCGGCTCAGGACTGAGG-3’. The amplification products from exon 3 were reamplified using the following sense nested primer 5’-CTCTCTGGCTGGC-3’. Annealing temperatures were 1°C below the lower primer melting temperature, as calculated using Gene Jockey software (Biosoft, Cambridge, UK, version 1.20). The PCR products were electrophoresed on 1% agarose gels. The resulting bands were excised, purified using a Qiaex Kit (Qiagen, Chatsworth, CA, USA), and sequenced directly using the Sequenase 2.0 Kit (US Biochemicals, Cleveland, OH, USA) following protocols recommended by the manufacturers. The products were electrophoresed on 6% polyacrylamide gels under denaturing conditions. The sense primers used for direct sequencing of enzymatically amplified exon 2 were: 5’-CGCGCATGTCAAGACC-3’, 5’-ATGGA-ACCTGACTTGTGTA-3’ and 5’-AGAACATGCTGGACTGTA-3’. Antisense sequencing primers for exon 2 were: 5’-GTCTAGCTGCTGCTGCAGCCGCGG-3’, 5’-AAGTCACCTCCACGTTGGTGT-3’ and 5’-AGACAGTGACAGGTCCCAT-3’. The exon 3 amplification primers were also used as sequencing primers for this exon.

**Loss of heterozygosity determinations and documentation of p53 mutations**

Loss of heterozygosity and p53 mutation analyses were reported previously in these tumour samples (Wan et al., 1994; Zheng et al., 1995).

**Statistical analyses**

Differences between two proportions were calculated using Fisher’s exact test. All P-values are double-sided.

**Results**

We sequenced the entire coding region of the **WAF1/CIP1** gene in 36 ovarian carcinomas. Such mutations were not found in any of these tumours, which included 18 carcinomas not harbouring any p53 mutations and 14 with loss of heterozygosity on chromosome 6p (Table I). A single nucleotide substitution, involving codon 31 of the **WAF1/CIP1** gene (AGC to AGA), was observed in 12 (33%) tumours (Table I). This substitution, changing the predicted amino acid from serine to arginine, was concordant in the
patients' normal cells and therefore constitutes a polymorphism rather than a mutation, in agreement with Chedid et al. (1994). Six of the 12 cases showing this polymorphism contained p53 mutations and four showed loss of heterozygosity on chromosome 6p. Thus, there was no correlation between presence of the polymorphism and either p53 mutations (P=1.3) or allelic loss on chromosome 6p (P=0.7). We sequenced 27 blood DNA samples obtained from normal individuals to determine whether this polymorphism was associated with increased cancer predisposition. The Arg31 allele was found in 10 of the 27 (37%) normal individuals, indicating that its frequency in the normal population was not different from that of patients with ovarian carcinomas (P=0.8).

Discussion

The results of our experiments clearly show that WAF1/CIP1 mutations are rare in ovarian carcinomas, including those tumours with allelic losses at the WAF1/CIP1 locus and those tumours without p53 mutations. These results are therefore not compatible with the idea that mutational inactivation of the WAF1/CIP1 gene is important for the control of ovarian tumorigenesis. Recently published studies (Shihohara et al., 1994; Li et al., 1995; Marchetti et al., 1995; Mousses et al., 1995) also failed to detect any mutational inactivation of the WAF1/CIP1 gene in different tumour types, including ovarian carcinomas. Thus, WAF1/CIP1 mutations may not constitute an important mechanism of tumour development in general, in spite of the fact that this gene is a downstream mediator of p53 activity.

The absence of WAF1/CIP1 mutations in ovarian tumours with losses of heterozygosity on chromosome 6p suggests that such losses may target a separate tumour-suppressor gene on this chromosome. Another possibility is that partial loss of WAF1/CIP1 activity or expression, either through unilateral allelic deletions or other mechanisms, may be sufficient to contribute to tumour development. In that regard, we tested the hypothesis that a recently described sequence polymorphism in the WAF1/CIP1 gene could be associated with ovarian carcinoma predisposition. We reasoned that this polymorphism could result in significant alterations in WAF1/CIP1 activity because it affects a conserved region of the gene and results in a major change in the predicted amino acid sequence (serine to arginine). However, there was no detectable difference in the frequencies of this polymorphism when patients with ovarian carcinomas were compared with a group of normal individuals. Likewise, there was no association between the presence of the variant allele and either p53 mutations or loss of heterozygosity on chromosome 6p. These results differ from those of Mousses et al. (1995), who observed an increased frequency of the variant allele in tumours without p53 mutations as well as a decrease in the frequency of this allele in tumours with p53 mutations. The reasons for these apparent discrepancies are not clear.

Table I Absence of WAF1/CIP1 mutations in 36 ovarian tumours characterised for the presence or absence of loss of heterozygosity (LOH) on chromosome 6p and of p53 mutations

| Case no. | p53 mutation | LOH on 6p | WAF1/CIP1 mutation | Codon 31 allele |
|----------|--------------|-----------|--------------------|----------------|
| 10       | CGT to GGT (codon 273) | No | None | Ser |
| 12       | None detected | No | None | Ser |
| 15       | None detected | Yes | None | Ser |
| 16       | ATC to ACC (codon 232) | No | None | Ser |
| 24       | GCC to CCC (codon 138) | Yes | None | Ser |
| 30       | None detected | No | None | Arg |
| 31       | CGT to CAT (codon 273) | Yes | None | Ser |
| 33       | CGC to CAC (codon 175) | No | None | Arg |
| 41       | CGT to CAT (codon 273) | No | None | Ser |
| 43       | None detected | No | None | Arg |
| 44       | None detected | No | None | Arg |
| 47       | TGC to TGG (codon 176) GAG to GAC (codon 271) | Yes | None | Ser |
| 51       | Loss of A (codon 209) | Yes | None | Ser |
| 53       | Immunopositive | No | None | Ser |
| 54       | None detected | No | None | Arg |
| 55       | None detected | No | None | Ser |
| 57       | None detected | No | None | Ser |
| 59       | Immunopositive | No | None | Ser |
| 61       | None detected | No | None | Ser |
| 62       | None detected | No | None | Ser |
| 63       | GTG to TTG (codon 173) | Yes | None | Arg |
| 64       | AGA to ATA (codon 209) | No | None | Arg |
| 67       | None detected | Yes | None | Ser |
| 69       | CGG to GGG (codon 248) | Yes | None | Ser |
| 71       | Immunopositive | Yes | None | Arg |
| 72       | None detected | Yes | None | Arg |
| 73       | Immunopositive | No | None | Ser |
| 74       | GTG to ATG (codon 272) | Yes | None | Set |
| 76       | None detected | Yes | None | Ser |
| 78       | None detected | No | None | Arg |
| 84       | None detected | No | None | Ser |
| 87       | None detected | No | None | Ser |
| 92       | TGC to TTC (codon 242) | Yes | None | Arg |
| 97       | CGG to TGG (codon 248) | No | None | Arg |
| 98       | None detected | No | None | Ser |

* Tumour with no detectable p53 mutation based on DNA sequencing studies but showing immunopositivity with anti-p53 antibody (Zheng et al., 1995).
One possibility is that these authors studied different tumour types including tumours of mesenchymal origin but did not examine ovarian carcinomas. In addition, it is not known if the p53 mutations that they examined were similar to those present in our tumour population.

There is growing evidence supporting the notion that derangements in cell cycle control play an important role in tumour development (Hartwell and Kastan, 1994; Hunter, 1993). Further evidence for such derangements in ovarian tumours comes from the apparent constitutive expression of the RB gene in these tumours (Kim et al., 1994; Dodson et al., 1994). Mutations in the p53 gene, which are frequent in ovarian tumours (Zheng et al., 1995), may account for such expression in some cases. Structural abnormalities in one or several other mediators of cell cycle activity must be present in the remaining cases. However, mutations in the RB gene itself are infrequent in these tumours (Kim et al., 1994; Dodson et al., 1994). The p16 gene, which is another important mediator of cell cycle activity involved in genesis of many tumour types (Kamb et al., 1994), is also intact in the same ovarian tumours (unpublished observations from our laboratory). Our above results suggest that the WAF1/CIP1 gene is likewise unaltered. Thus, the underlying mechanism(s) leading to cell cycle derangements in ovarian carcinomas not harbouring p53 mutations is/are still unclear.

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