

# Aberrant DNA methylation of *p16* onco-suppressor gene in human cervical carcinoma

## Research Article

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## Summary

The aim of this paper is to investigate the role played by DNA methylation in uterine cervical carcinoma. Since in a subset of human cancers the onco-suppressor *CDKN2/p16* gene is an essential target for malignant transformation, its first and second exon regions were investigated for their DNA methylation levels by PCR methylation-dependent analysis. Our results on purified DNAs from cervical tissues show that, in spite of the diffuse DNA hypomethylation which characterizes neoplastic cells, specific DNA methylation of HpaII and CfoI sequences of the first exon occurs and increases with the grade of neoplastic transformation. These data support the idea that the *p16* onco-suppressor gene is directly involved in malignant transformation through the methylation process.

## I. Introduction

DNA methyltransferase(s) by transferring methyl groups from S-adenosyl methionine to cytosines (Adams and Burdon, 1985) - which are essentially located in the CpG dinucleotides - encodes epigenetic information on DNA of great importance for the control of gene expression (Ng and Bird, 1999, Bird AP and Wolffe AP, 1999). The distribution of cytosines and 5-methylcytosines is non-random in genomic DNA and the methylation pattern, defined in early stages of embryonic development (Brandeis et al, 1993), is that in which methylated cytosines are present in the bulk of DNA while some DNA regions (1-2%) of about 1-2 Kbp in size, termed CpG islands, result in being highly protected from methylation despite their enrichment in dinucleotide CpG by about 5-10 times. The maintenance of this unmethylated state is extremely important since these DNA regions are located in the promoter regions of

housekeeping genes and there is evidence that transcription of genes correlated with CpG islands is inhibited when these regions are methylated (Bird, 1986; 1987). CpG islands have also been found to overlap part of the gene but in this case their methylation is not related to gene silencing (Jones, 1999).

The DNA methylation process, through DNA methylase (Bestor and Ingram, 1983; Okano et al, 1998; Okano et al, 1999) and demethylase activities (Bhattacharya et al, 1999; Ramchandani et al, 1999), is involved in carcinogenesis in a paradoxical way. It is in fact possible to identify the development of two contrasting events in the same tumor sample: a general pattern of DNA hypomethylation (Feinberg and Vogelstein, 1983; Gama-Sosa et al, 1983; Goelz and Vogelstein, 1985; Feinberg et al, 1988; Kim et al, 1994; Laird and Jaenisch, 1994; Jurgens et al, 1996; Bernardino et al, 1997; de Capoa et al, 1999; Soares et al, 1999) in which specific onco-suppressor genes become methylated

(Herman et al, 1994, 1995; Merlo et al, 1995; Fueyo et al, 1996; Nuovo et al, 1999; Baylin and Herman, 2000; Costello et al, 2000).

Presently the high level of DNA demethylase found in neoplastic cells (Ramchandani et al, 1999; Bhattacharya et al, 1999) explains the spreading of DNA hypomethylation during tumorigenesis. However the aberrant hypermethylation of onco-suppressor genes, which would seem to be one of the most important events involved in tumorigenesis, still cannot be explained (Bird and Wolffe, 1999).

An interesting – but as yet undemonstrated model – has been put forward (Baylin, 1997) to account for this hypermethylation of onco-suppressor genes in tumor cells. The fact that interaction between PCNA and DNMT1 is found in transformed cells (Chuang et al, 1997), is the basis of this model which supposes that a cellular event – to be identified – allows the DNMT1 to bind PCNA in an early replication phase and this would make it possible for the enzyme to methylate the DNA when CpG rich DNA regions replicate themselves (Selig et al, 1992).

Thinking about this paradoxical behaviour of DNA methylation in neoplastic cells, the purpose of our preliminary studies was to analyze the DNA methylation pattern in human cervical tumors, taking the onco-suppressor gene *CDKN2/p16* (Bonetta, 1994; Marx, 1994) as a model.

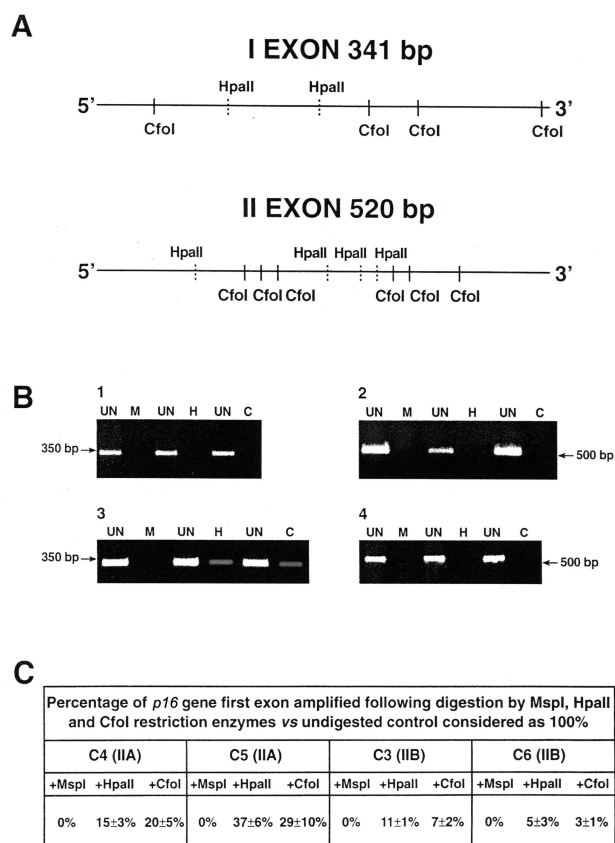
The *CDKN2* gene, located in chromosome 9p21 (Kamb et al, 1994), is likely to be a tumor suppressor gene since lack of its expression is frequently observed in many types of cancers (Nobori et al, 1994; Gonzalzo et al, 1995, 1997; Maesawa et al, 1996). This gene encodes the p16 protein (16 KDa), which plays in normal cells a critical role in cell-cycle regulation. In fact the p16, through its association with the cyclin-dependent kinase-4, inhibits kinase activity and consequently the progression from G1 to the S phase of cell cycle.

## II. Results and Discussion

### A. Evaluation of *p16* gene methylation pattern

The evaluation of *p16* gene methylation level was carried out by the PCR-based methylation assay (Gonzalzo et al, 1998). This method foresees the digestion of DNA samples by methylation-sensitive restriction enzymes before PCR amplification, so that the DNA fragment will be amplified only if the enzymes do not cut inside the sequence. Enzymes used in the experiments were HpaII, CfoI and MspI. By comparing the electrophoretic pattern of non-digested amplified fragment to those obtained following enzymatic digestion we came up with information about the state of methylation of CpG moieties in the restriction sites. It is important to remember that more than one HpaII and CfoI site (Figure 1A), is located in the first and second *p16* exon so that, since amplification occurs only if all sites are methylated

the low level of amplification product depends on the fact that not all sites are totally methylated. As shown in Figure 1B (1, 2), when digestions were carried out on DNA samples from healthy tissues no PCR amplification of DNA fragment either from the first or the second exon of *p16* gene was found. These data show that in normal tissues and at least for the specific sequences examined, both exons were unmethylated. A different result was obtained by performing the same experiments on DNA purified from tumor samples. In Figure 1B (3, 4) the electrophoretic pattern of one tumor sample (C4), corresponding to an advanced stage (IIA) of cervical



**Figure 1:**

**A** - maps showing the HpaII (MspI) and CfoI sites in the first and the second exon of *p16* gene.

**B** - PCR amplification pattern of the first (1, 3) and the second exon (2, 4) of *p16* gene from healthy (F2) and carcinogenic (C4) samples: UN (undigested control), M (digestion by MspI), H (digestion by HpaII), C (digestion by CfoI).

**C** - table showing the results of densitometric scanning (Bio Image, Millipore) of the bands of *p16* gene first exon amplified from tumor tissues (C3, C4, C5, C6) following digestion by methylation sensitive restriction enzymes. The average value of four independent experiments is presented.

cancer is shown. In these experiments DNA samples were digested by three different restriction enzymes before PCR

amplification. Following HpaII and CfoI digestion a partial amplification was observed in the first but not in the second exon. When stage IB samples were examined result was different because there was either no amplification (C1, C2) or a lower amplification level (C3, C6). **Figure 1C** shows densitometric scanning of the electrophoretic bands obtained following digestion and PCR amplification of tumor samples (C3, C4, C5, C6). These data are indicative of an anomalous methylation pattern in the first exon of *p16* gene in tumor DNA. As far as the second exon is concerned, since the methylation dependent assay is a method in which the amplification occurs only if all sites are methylated, we cannot exclude that some of the numerous HpaII or CfoI sites in the second exon are partially methylated.

### B. Evaluation of DNA methylation level by methyl-accepting ability assay

The methylation level which characterizes the neoplastic vs normal epithelial cervical cells, was evaluated by DNA methyl-accepting ability assay carried out on purified DNAs. As shown in **Figure 2**, all tumor samples showed higher levels of methyl-accepting ability, about 2.5-5 times more than in normal samples, thereby indicating lower levels of pre-existing methyl groups onto DNA.

Our results have shown that despite the general DNA hypomethylation that characterizes the neoplastic cells, in 66% of the neoplastic samples examined there is an anomalous presence of some methyl groups on the HpaII and CfoI sensitive sequences located in the first exon of *CDKN2/p16* gene. Indeed, it is interesting to note the co-existence in the same DNA sample of a general hypomethylation and a specific onco-suppressor gene hypermethylation.

However it must be remembered that these two phenomena do not co-exist in the DNA from all types of tumors since while diffuse DNA hypomethylation has been shown to be a general state of neoplastic cells (Feinberg and Vogelstein, 1983; Gama-Sosa et al, 1983; Goelz and Vogelstein, 1985; Feinberg et al, 1988; Kim et al, 1994; Laird and Jaenisch, 1994; Jurgens et al, 1996; Bernardino et al, 1997; de Capoa et al, 1999; Soares et al, 1999) the anomalous hypermethylated state of specific onco-suppressor genes has not been identified in all kinds of tumors (Soares et al, 1999). For example in human urothelial carcinoma, the co-existence of hypo and hypermethylation, observed in tumor cell lines, is not present in tumor tissues where only general DNA hypomethylation is evidenciabile (Soares et al, 1999).

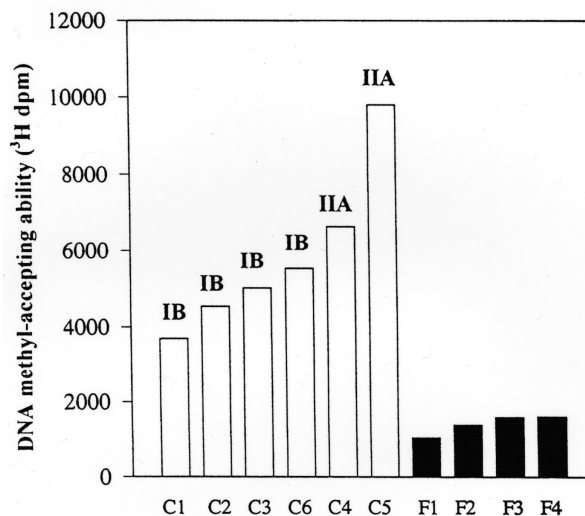
Therefore although the correlation between DNA methylation and neoplastic evolution would seem to be evident (Jones et al, 1992; Laird and Jaenisch, 1994; Laird and Jaenish, 1996; Schmutte and Jones, 1998; Jones and Laird, 1999; Jones, 1999) it is still difficult to establish whether these changes in methylation level are involved in

inducing cell transformation or if they play a supporting role in the progression of carcinogenesis.

### IV. Materials and Methods

Experiments were performed on tissue samples from six patients affected by pathologically proven cervical squamous carcinoma. Mean age was 60 years (range: 50 - 75). According to the current FIGO staging system 4 patients were assigned to IB (3 IB1 and 1 IB2) and the other two women to IIA stage of disease. An adequate amount of cervical tissue was biopsied directly from the tumor mass: half of the specimen was referred to the pathologist to confirm the diagnosis, while the other half was processed. A control group of 5 healthy women, all having cytological and colposcopic negative tests, underwent cervical punch biopsy. Tissue samples were divided into two parts, one was analysed by the pathologist and the other was processed in our experiments.

Each DNA sample (2 µg in 160 µl), from normal and malignant tissues, was pre-digested with 10 units of EcoRI at 37°C for 6 hours with the aim to improve the efficiency of enzymic digestion. Subsequently each sample was divided in four aliquots (40 µl) and DNAs digested by adding methylation independent enzyme as MspI and methylation dependent enzymes as HpaII and CfoI; the fourth aliquot was not added of any enzyme representing the undigested control. The enzymic digestion was carried out at 37°C for 36 hours and 10 units of enzyme were added every twelve hours for a total amount of 30 units of enzyme. PCR amplification was performed with the aim



**Figure 2:** Methyl-accepting ability of DNAs extracted and purified from differently staged tumor samples (C1 IB, C2 IB, C3 IB, C4 IIA, C5 IIA, C6 IB) and from normal samples (F1, F2, F3, F4).

of amplifying the first and the second exon of onco-suppressor gene *p16* (Gonzalzo et al, 1998).

Primers used for amplification of the first exon were: forward 5' GAAGAAAGAGGAGGGGCT 3' and reverse 5' GCGCTACCTGATTCCAAT 3'. Primers used for amplification of the second exon were: forward 5' GGAAATTGGAAACTGGAA 3' and reverse 5' AAAATGAATGCTCTGAGC 3'.

The PCR mixture contained 100 ng of DNA (8 µl of digestion solution), primers (50 pmol both in forward and in reverse), dNTPs (final concentration of 0.2 mM) and 2.5 units of *Taq* DNA polymerase in Qiagen amplification buffer without MgCl<sub>2</sub>. The final concentration of MgCl<sub>2</sub> was of 1.4 mM and it was added into reaction mixture from a 25 mM stock of MgCl<sub>2</sub> taking into account the drawn volume and the concentration of MgCl<sub>2</sub> present into buffer we used for restriction enzymic digestions. The reaction (50 µl) was carried out under the following conditions for both exons: denaturation at 95°C for 5 min; 95°C for 1 min, 55°C for 1 min, 72°C for 1 min for 30 cycles and a final segment at 72°C for 6 min. The amplified fragments were evidenced by 2% agarose gel.

DNAs purified from different sample tissues were used as substrate for evaluating their methyl accepting ability. In a final volume of 100 µl 2.5 µg of DNA were incubated in presence of 1.5 units of bacterial SssI methylase using as methyl donor 80µM S-adenosyl-methionine plus 30 µCi /ml [<sup>3</sup>H] S-adenosyl-methionine for 1 hour at 37°C. The reaction was stopped by addition of 1% SDS and 250 µg/ml of proteinase K at 37°C for 30 min. After cooling on ice 100 µg/ml of salmon sperm DNA were added as carrier and DNA was precipitated at 0°C with 20% of trichloroacetic acid (final concentration) and centrifuged at 7000 rpm for 10 min. Pellets were washed with 5% trichloroacetic acid and resuspended in 0.5 ml of 0.5 M NaOH and heated at 60°C for 30 min. After cooling in ice, DNAs were precipitated with 15% trichloroacetic acid and then each DNA sample was recovered on a glass fiber paper (GF/C Whatman). The filters were repeatedly washed by 5% trichloroacetic acid and by 95% ethanol. The incorporated radioactivity was measured in a Beckman Ls-6800 liquid scintillation spectrometer.

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