Exon 4 and intron 4 *TP53* are both methylated in advanced-stage ovarian carcinomas

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Abstract. Although intragenic CpG dinucleotides are conserved during evolution, they are also sensitive to methylation-dependent mechanisms. Methylation status of the *TP53* introns 1, 3 and 4 have been analysed in stage III ovarian carcinoma (OC). In the present study, the methylation of exon 4 (10 CpG pairs) was analysed in advanced-stage OC to investigate *TP53* methylation and compare exon and intron 4 methylation patterns. A total of 80 samples from patients with advanced-stage OC and metastatic lesions were examined, along with 80 samples derived from healthy patients who had never been diagnosed with cancer. Methylation analysis of the human A2780 ovarian cancer cell line was also performed. Exon and intron 4 were methylated in OC, corresponding metastases and paired healthy tissue. The DNA from the human A2780 ovarian cancer cell line and the normal samples from healthy subjects was also methylated. The data indicate the existence of an intragenic mechanism of regulation of *TP53* activity that involves demethylation/methylation processes. This mechanism provides the ability to alter the response from cell cycle arrest to apoptosis by manipulating only the expression of long or short p53 isoforms.

Introduction

Epigenetic DNA modification mechanisms play a key role in various cell cycle functions (1,2). High methylation of certain fragments or whole chromosomes may be associated with partial or complete transcriptional inactivation. A total of ~5% of cytosine residues are continuously methylated in mammals (3). The methylation percentage differs between species; for example it is often 30% in plants but does not appear in *Drosophila melanogaster* (4).

In mammals, introns constitute up to 95% of the primary gene transcripts (5). Although the introns do not encode proteins, they participate in important cellular functions (6). Firstly, introns enhance the expression of corresponding genes and must be present in the transcribed region to enhance gene expression (7,8). Secondly, the introns increase transcript initiation upstream and may represent a downstream regulatory element for genes transcribed by RNA polymerase II (8).

DNA methylation mechanisms have been reviewed in the context of ovarian cancer development and progression (1,9). However, a limited number of studies have analysed the CpG island *TP53* methylation status at the promoter region and within introns (10,11). For example, the *TP53* promoter is methylated in 51.5% of ovarian carcinoma (OC) samples and 29.7% of patients with healthy ovaries. However, no clinicopathological parameters are associated with the *TP53* methylation pattern. These data revealed a significant difference in promoter *TP53* methylation between OC and control samples, implying the influence of *TP53* methylation on ovarian tumorigenesis (10).

In our recent study, the *TP53* methylation status was investigated in a cohort of 80 patients with stage III OC. Intron 1 was un-methylated in all samples, whereas introns 3 and 4 were methylated (12). In the present study, 10 CpG exon 4 pairs were analysed in primary OC, corresponding metastases, healthy samples and the A2780 ovarian endometroid adenocarcinoma. The aim of the present study was to compare the *TP53* methylation status of exon and intron 4 in advanced-stage OC samples.

Materials and methods

Samples. The tissue samples were collected from 80 patients aged 55-65 who had undergone surgical treatment for advanced-stage OC at the Department of Gynecology and

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Abbreviations: BOT, borderline ovarian tumor; ncRNA, non-coding RNA; OC, ovarian carcinoma

Key words: TP53, methylation, ovarian cancer, exon 4, intron 4

Gynecology Oncology at the Military Institute of Medicine, Warsaw, Poland, between January 2014 and December 2018. The present study included only patients with FIGO (International Federation of Gynecology and Obstetrics) stages IIIA-C (13).

Chemotherapy, hormonal therapy or radiotherapy were not administered prior to the operation. In total, the study included samples from 40 patients with serous G2/3 and 40 patients with endometrioid G2/3 OC, corresponding metastatic samples and healthy tissue (skin) samples from each patient. The detailed clinicopathological features of the patients have been previously presented (12). The human ovarian cancer A2780 cell line (Merck KGaA), established from an ovarian endometrioid-type adenocarcinoma in an untreated patient, was also included. Control samples (skin) were sourced from 80 patients aged 35-45 years (50% male and 50% women), who had never had cancer who and underwent bariatric surgical operations at the Department of Surgery, Military Institute of Medicine, Warsaw, Poland, between January 2014 and December 2018. The present study was approved by the Bioethics Committee of the Military Institute of Medicine, Warsaw, Poland with the informed consent of the patients (approval no. N25/WIM/2013).

Following fixation in 10% buffered formalin (pH 7.4) at room temperature for 24 h, hematoxylin and eosin-stained slides (room temperature for 1 h) were prepared from paraffin-embedded tissue (12). Thickness of sections were 4 μm. The slides were analysed with light microscope by the anatomopathologist to confirm the primary diagnosis (14).

DNA isolation and bisulfite conversion. Total genomic DNA was isolated using the ExtractMe DNA Tissue Isolation kit (cat. nr 51404; Qiagen GmbH, Germany). The quantity and quality of DNA was evaluated spectrophotometrically (DeNovix DS-11; DeNovix Inc.). Bisulfite DNA conversion was performed using the Methyl Code Bisulfite Conversion kit (cat. nr. 1024702; Invitrogen; Thermo Fisher Scientific, Inc.) as previously reported (12).

Primer sequences. Gene-specific primer sequences for exon 4/intron 4 were designed based on the *TP53* sequence published in National Center for Biotechnology Information (NC_000017.10) using MethPrimer-Design software version no. 1 (urogene.org.) (15). Primer sequences were as follows: Forward, 5'-TTGTGTAGTTGTGGGTTGATTTTATAT-3', and reverse, 5'-AAAAACCTAAAAACCCTAAACAACC-3'. The product size was 193 bp. In total, 11 CpG pairs were investigated, of which one spanned the *TP53* intron 4 and 10 the *TP53* exon 4 (Fig. 1).

DNA amplification and sequencing. The amplification of the *TP53* exon 4/intron 4 sequences was performed using MyTaqHS Red Mix (cat. no. BIO-25048; Blirt) as described previously (12). Thermocycling conditions were as follows: Initial denaturation at 95˚C for 1 min, followed by denaturation at 95˚C for 15 sec, annealing at 56˚C for 15 sec, extension at 72˚C for 15 sec, number of cycles 45, and final extension at 72˚C for 4 min. PCR products were verified by electrophoresis on 1.5% agarose gels (1 ug/line) using Midori Green nuclear staining dye (cat. no. MG04; Nippon Genetics Europe GmbH). The product was extracted for cloning with the use of ExtractMe DNA Gel-out kit (cat. no. 28706; Qiagen GmbH). The ligation between the plasmid vector and the PCR product was performed using Qiagen PCR Cloning kit (cat. no. 231124; Qiagen GmbH). Finally, the vectors were transformed into *E. coli* high efficiency competent cells according to the manufacturer's instructions (New England BioLabs Ltd.). The cells were incubated at 37˚C overnight on LB Agar Miller (Medium A&A Biotechnology) with ampicillin, isopropyl-β-1-thiogal actopiranozyd) and X-gal (cat. no. MBO2501; Blirt, Poland). A blue-white screening colony selection method was used to select a recombinant white clone followed by PCR amplification (as aforementioned) with MyTaqHS Red Mix (cat. no. BIO-25047; Meridian Biosc., USA) of the colony to confirm the cloning with the gene segments of interest. Only white colonies of recombinant plasmid isolation from the bacteria growing in liquid Luria Broth medium plate were selected and cultured in LB medium at 37˚C overnight. The plasmid was isolated using Plasmid Mini DNA Isolation kit (cat. no. 020-50 A&A Biotechnology, Poland). The results of the recombinant DNA were examined using 1.5% agarose gel electrophoresis (1 μg/lane). The clones containing right inserts were subjected to direct bidirectional sequencing using an AB3130 genetic analyser with T7/SP6 primers and an Big Dye® Terminator v3.1 Cycle Sequencing kit (cat. no. 4337455; Thermo Fisher Scientific, Inc.). All KITs were used according to the manufacturer's instructions.

Results

Exon 4 *TP53* CpG pairs were all methylated in neoplastic ovarian samples collected from patients with advanced-stage OC and in the corresponding metastatic samples. The tissue samples from healthy people who had never had cancer were also all methylated. An example of the *TP53* exon 4 methylation in the sequencing samples of primary stage IIIC endometrioid-type OC is presented in Fig. 2.

Similarly, one intron 4 *TP53* CpG pair revealed methylation in all samples. Moreover, the A2780 human ovarian cancer cell line revealed *TP53* exon 4/intron 4 methylation (data not shown).

Finally, no differences were noted in the exon/intron 4 methylation statuses of *TP53* based on clinicopathological OC features (Table I).

Discussion

DNA methylation serves a crucial role in normal embryonic development, aging, gene regulation and specific cells functions (16). In general, CpG islands located within gene promoters are unmethylated in normal human cells, except for the inactive genes spanning the X chromosome and those subjected to genomic imprinting (the process where one paternal copy of the certain gene is silenced) (16,17). However, silencing of tumour suppressor gene activity in most cases occurs through methylation of promoter regions at all stages of human carcinogenesis, including the development of OC (1,2,9-12,17).

Figure 1. Sequence of intron (yellow) and exon 4 of *TP53*. Red, CpG. >, forward primer; <, reverse primer; + presence of cytosine methylation, : absence of cytosine methylation; |, nucleotide reference.

Figure 2. *TP53* exon 4 methylation in sequencing sample of primary advanced-stage endometrioid-type ovarian carcinoma.

Table I. Clinical and pathological variables of patients with ovarian carcinoma.

Characteristic	Samples (%)
Age, (years)	
<50	1 (1.5)
50-60	18 (22.5)
>60	61 (76)
Menopause status	
Pre-menopause	3 (4)
Post-menopause	77 (96)
Clinical stage	
IIIA	5 (6)
IIIB	36 (45)
IIIC	39 (49)
Histological type	
Serous	40 (50)
Endometrioid	40 (50)
Histological grade	
G1	0 (0)
G2	37 (46)
G3	43 (54)

OC is one of the most insidious and dangerous female genital tract malignancies due to its high aggressiveness (18,19). The majority of OC is reported at advanced clinical stages of the disease. The 5-year survival rate of stages III and IV is still unsatisfactory, despite the use of extensive surgical procedures and the development of anti-cancer therapy treatments (20,21). The pathogenesis of OC is still debatable. Kurman and Shih (22) proposed that fallopian tube epithelium (benign or malignant) that implants on the ovary is the source of low- and high-grade serous carcinoma rather than the ovarian surface epithelium. Two OC histological subtypes (type I, low-grade serous or endometrioid, clear cell, mucinous and transitional OC, and type II: high-grade serous, undifferentiated, and carcinosarcoma) differ significantly regardless of the *TP53* alterations and p53 immunoreactivity (23). Different *TP53* alterations and p53 expression patterns have been described in human borderline ovarian tumours (24). However, data regarding the role of *TP53* exogenic/intragenic methylation status during ovarian carcinogenesis are scarce (9-12,25).

Although the role of extragenic methylation on *TP53* has not been fully resolved, CpG pairs are vulnerable to methylation/demethylation mechanisms (7). Altered exonic CpG methylation modifies promoter initiation sites, resulting in the expression of different protein isoforms (26,27). Exogenic CpG sequences in *TP53* are methylated in various cancer types (for example colon and lung cancer) (25). Genetic *TP53* alterations are associated with transitions (G:C->A:T), which are frequently found in human tissue and normal cell lines, for example in lung epithelial cells, mammary epithelial cells, or in colonic mucosa cells (25) Moreover, non-CpG methylation in CC and CCC sequences is associated with methylation at repetitive *TP53* genetic sequences (28).

Hydrolytic deamination of 5-mC is typically considered as the mechanism responsible for the high incidence of *TP53* C-T transition mutations within CpG dinucleotides (3,25). C-T transitions at CpG may also result from methyltransferase-catalysed cytosine deamination. The frequency and types of *TP53* mutations at CpG dinucleotides vary between human tumours (1-3,23). For example, in colon carcinoma, 47% of mutations are reported at CpG islands, with 17% are reported in skin cancer and 9% in lung cancer (1-3).

Although the methylation status of introns 1-4 in *TP53* in advanced-stage OC has been previously studied (12), to the best of our knowledge, exon 4 has not been previously investigated. The present study examined exon/intron 4 *TP53* and demonstrated that 11 CpG islands were all methylated. Most studies that have examined comprehensive DNA methylation have focused on the promoter regions of genes, and, in the majority of the cases, an inverse association between gene expression and methylation has been found (1,3,25). Methylation in downstream exon sequences generally is not associated with expression or lack of expression of p53 in various tissues. The *TP53* sequences along exons 5-8 are completely methylated at each CpG, including 46 different sites on both DNA strands (25). This methylation pattern is tissue-independent, suggesting that tissue-specific methylation does not contribute to the differential mutation pattern in various tumours. A total of nine types of normal human tissues and cell lines, including skin fibroblasts, keratinocytes, lung and mammary epithelial and colonic mucosa cells, have been investigated (25). However, it is unclear whether complete methylation of all CpG sites is unique to *TP53*.

Although *TP53* CpG dinucleotides are prone to methylation-dependent mechanisms (25), the regulatory role of methylation mechanism affecting CpG sites has yet to be clarified. In previous studies, DNA damage and cell aging are both associated with site-specific CpG demethylation in exon 5 accompanied by the induction of expression of truncated protein isoforms regulated by an adjacent intronic P2 promoter (spanning intron 4) (25,26). The changes in the levels of intragenic *TP53* CpG methylation are extrinsically inducible, suggesting

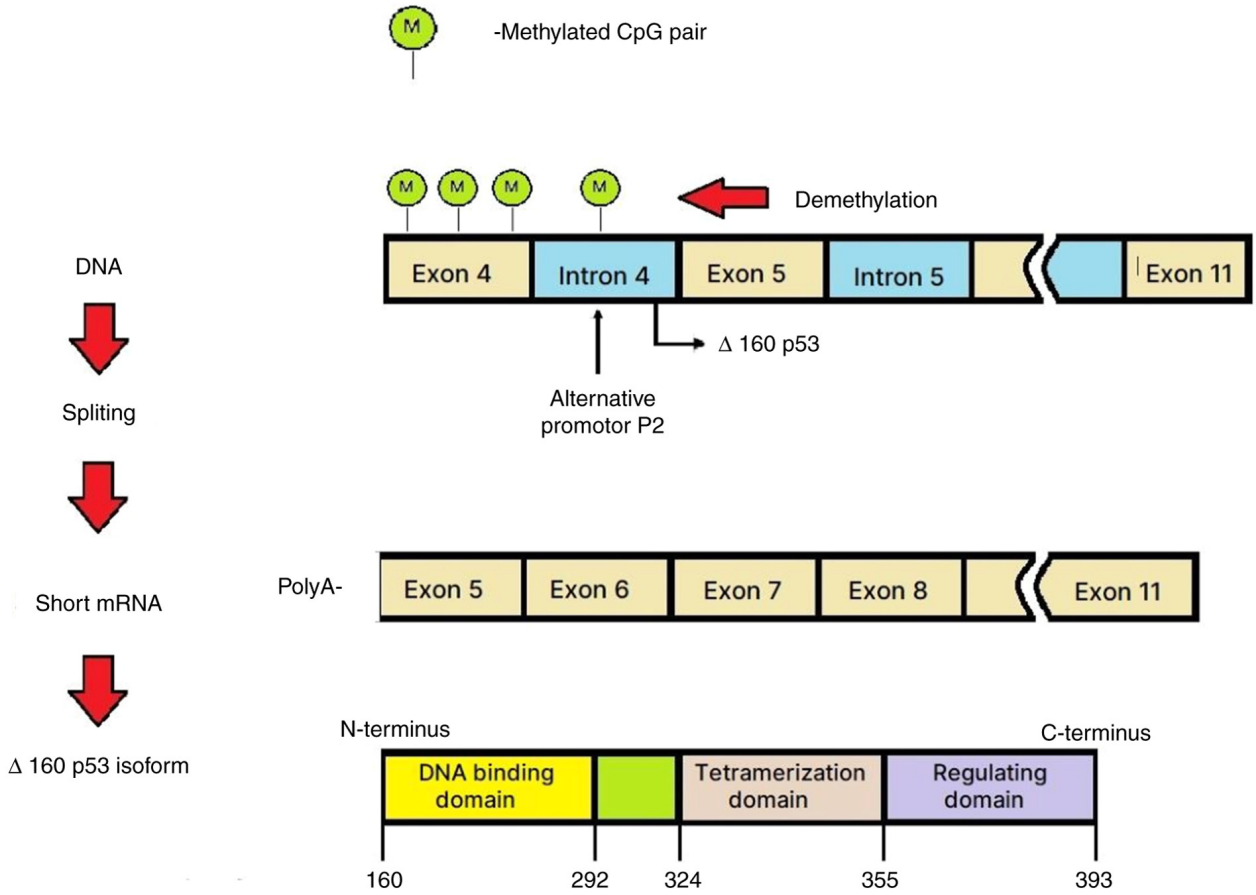

Figure 3. Intragenic demethylation/methylation mechanisms regulating *TP53* activity.

that cancer progression is mediated, in part, by dysregulation of damage-inducible intragenic CpG demethylation (26).

The role of alternative promoters in mammalian genomes has been reviewed by Landry *et al* (27). Bourdon *et al* (28,29) reported that *TP53* has transcriptional start sites that span exon 1 and contain a transcription initiation site at intron 4. The aforementioned study revealed that *TP53* had a complex transcriptional regulatory pattern encoding different p53 mRNA variants through the use of alternative splicing and an internal (intron 4) promoter. The alternative promoter leads to the expression of N-terminally truncated proteins. The two distinct *TP53* promoters (P1, upstream of exon 1, and P2, within intron 4) and alternative splicing process and translation initiation sites of the different mRNAs result in formation of various p53 isoforms (30).

The transcription of *TP53* mRNA can initiate the formation of the Δ133p53 and Δ160p53 isoforms from the internal P2 promoter. Considering alternative splicing at intron 9, these transcripts lead to the production of various isoforms (Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β and Δ160p53γ) (31). Despite truncations in the DNA binding domain, the Δ133p53 and Δ160p53 isoforms have a stable 3D conformation (31). The alternative promoter located in intron 4 becomes active following DNA demethylation in this region. Therefore, the generated p53 isoforms are shorter, lack the mouse double minute homolog 2 binding site, have a longer life-span and can potentially induce apoptosis (28,29,32,33).

In conclusion, the present findings suggest the existence of intragenic mechanisms responsible for the regulation of the TP53 activity, based on demethylation/methylation status (Fig. 3).

The intragenic demethylation-methylation mechanism provides the ability to switch the cellular response from cell cycle arrest to apoptosis by manipulating only the expression of p53 isoforms and is damaged in solid cancer. Therefore, it has been hypothesized that demethylation of the *TP53* promoter in intron 4 may be a target for the potential treatment of solid tumours.

The present study had limitations. Firstly, other histopathological OC subtypes, apart from serous and endometrioid subtypes, should be investigated to explore the intron/exon 4 *TP53* methylation pattern. Secondly, survival of patients with OC in relation the *TP53* intron 4/exon 4 methylation was not analysed. Future studies should explore potential interactions between *TP53* methylation patterns and other epigenetic modifiers (such as histone modification). Moreover, *TP53* mutational analysis in advanced-stage OC should be performed.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WS conceived and designed the study, and performed the experiments. OS made substantial contributions to the conception of the study, design of the figures, and analysis and interpretation of the data. KC analyzed and interpreted the data. RG performed and analyzed the sequencing. MW participated in sample collection, and analysis and interpretation of the data. MS analyzed and interpreted the data. AS analyzed and interpreted the data, revised the manuscript and prepared the final version of the article. WS and AS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Bioethics Committee of the Military Institute of Medicine, Warsaw, Poland (approval no. N25/WIM/2013). All participants read and signed an informed consent form prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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