

p53 mutant His¹⁷⁵ identified in a newly established fallopian tube carcinoma cell line secreting interleukin 6

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Abstract Fallopian tube carcinoma is a lethal gynecologic malignancy. Etiologic factors are unknown. No experimental data on molecular alterations exist so far. For an in vitro model, we established the permanent human tubal carcinoma cell line FT-MZ-1. The median doubling time was 14 days with 24.2% in S phase. A point missense mutation of the p53 tumor suppressor gene resulting in the His¹⁷⁵ mutant was identified. Aberrant p53 protein accumulated in nucleus and cytoplasm. FT-MZ-1 substantially secreted interleukin 6 (IL-6) coinciding with the inactivation of p53 as a transrepressor on the IL-6 gene promoter.

Key words: Fallopian tube carcinoma; Tumor suppressor gene; p53 mutation; Interleukin 6; Proliferation

1. Introduction

Cancer of the fallopian tube is a disease comprising only 0.3 to 1.1% of all malignancies of the female genital tract [1,2]. One-third presents at a late stage with a 5-year survival of less than 10% [2]. Etiologic factors are not known; however, low conception rate and chronic salpingitis are commonly found conditions in patients who develop fallopian tube carcinoma. Only tumor stage has so far been shown to predict prognosis [3]. Molecular events such as mutational activation of oncogenes or inactivation of tumor suppressor genes associated with this cancer have not been reported so far.

Mutations of the p53 tumor suppressor gene are among the most common single-gene alterations associated with human malignancy identified so far [4,5]. Inactivation of the p53 gene may contribute to loss of a cell cycle check point at the G1-S boundary and to genetic instability of the cell eventually allowing cells to replicate in an uncontrolled fashion [6–9]. p53 inactivation may be induced by different genetic mechanisms leading to loss of a complete p53 allele and/or to subtle intragenic mutations both of which have been observed in epithelial ovarian cancer [10–16], with similarities to fallopian tube cancer in clinical behaviour and management.

We report on the first permanent cell line established from a human fallopian tube carcinoma and on associated molecular alterations. The entire p53 coding region was analyzed for mutational inactivation and its effect on p53 protein expression was studied. Interleukin 6 secretion, cytokeratin expression and proliferative properties of the cultured cells were determined.

2. Materials and methods

2.1. Cell culture

The cell line FT-MZ-1 was established from the solid primary tumor of a FIGO stage III fallopian tube carcinoma from a 52-year-old patient by techniques as previously described [17,18]. The tumor was histopathologically examined by two independent pathologists (H.E.R. and G.D.). FT-MZ-1 cells were kept in Dulbecco's modified Eagle medium

(DMEM) with 10% fetal calf serum (Gibco) and were analyzed at passage 18. Ovarian cancer cell line OV-MZ-7a presenting with a low IL-6 secretion level served as a control. Breast cancer cell line SK-BR-3 containing the His¹⁷⁵ mutant of the p53 gene was obtained from ATCC. Genomic DNA was isolated from the cell lines as well as from fresh placental tissue by standard procedures. The DNA sample D263 also containing the His¹⁷⁵ mutant was a generous gift from S. Baker and B. Vogelstein.

2.2. Growth rate and heterotransplantation

Doubling time and tumorigenicity were assessed as previously described [18].

2.3. Flow cytometry

FACScan analysis to determine S phase fraction and ploidy was carried out as previously described [19].

2.4. Analysis of cytokine secretion

Concentration of IL-6 secreted into the medium was analyzed by a solid phase EASIA kit (Medgenix, Bruxelles) after 4 days of log phase culture according to the manufacturer's protocol.

2.5. Heteroduplex (HD) and single-strand conformation polymorphism (SSCP) analysis

Screening for HD and SSCP as indicators for point mutational events was carried out using a high resolution polymer gel (Hydrolink-MDE, AT Biochem, Malvern, PA, USA/Serva, Heidelberg, Germany) according to manufacturer's recommendations. PCR primer sequences flanking exons 5 to 9 have previously been published [20]. PCR primers flanking exons 2 to 3, 4, 10, and 11 (5'-AGAATTCGATCCTCTTG-CAGCAGCCAGAC-3', 5'-AACCCCTGTCTTACCAGAAC-3', 5'-TTTACCCATCTACAGTCCC-3', 5'-ACCTAGGCTCAGGGCACTGACCG-3', 5'-GGGAATTCAGATCCGTGGGCGTGAG-3', 5'-GCTGAGGTCACTCACCTGGA-3', 5'-GCTTCTGTCTCCTACAGCCAC-3', 5'-ACCTAGGGCTGTCTAGTGGGAACAAGAA-G-3', respectively) were used in addition to screen the entire p53 coding region for heteroduplexes or SSCP. Placental DNA sample which contained wild-type p53 as previously confirmed by sequencing [21] was used as negative control. In heteroduplex analysis, placental DNA was also used to identify hemizygous point mutations. Prior to heat-denaturation an equal amount of placental PCR product was added to allow the formation of a possible heteroduplex between cell line and placental DNA strands after slow renaturation controlled by a thermal cycler.

2.6. Multiplex PCR (MPCR)

The entire protein coding region was PCR amplified using four sets of oligonucleotides in a single PCR preparation essentially as described

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previously [20,21]. Fragments were electrophoresed on a vertical Hydrolink-MDE gel (AT Biochem, Malvern, PA, USA/Serva, Heidelberg, Germany).

2.7. Nucleotide sequencing

For sequence analysis of exon 5 a cycle sequencing strategy was applied. For PCR and sequencing reaction priming, the following oligonucleotides were used: Sense primer #O-127 5'-GGAATTCCA-CACCCCCGCCG-3' and antisense primer #O-205 5'-CGGA-ATTGAGCGGCTCATAGGGC-3'. For template preparation, PCR products were directly purified on a spin column (Centricon-100, Amicon, Beverly, MA, USA). The sequencing reactions were prepared according to manufacturer's recommendations (Cyclist DNA sequencing kit, Stratagene, La Jolla, CA, USA) carried out in a thermocycler (Minicycler, MJ Research, Watertown, MA, USA) with an initial denaturation step of 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 54°C for 30 s and 70°C for 1 min. After thermal cycling, 1 µl of the reaction mix was added to 3 µl of sequencing stop solution and heat-denatured. For electrophoresis and blotting an Autotrans 350 direct transfer electrophoresis system (Betagen, Waltham, MA, USA) was used. One µl of the mix was loaded on a 35 cm long 0.2 mm thick 6% polyacrylamide gel containing 7.5 M urea. Electrophoresed fragments were blotted on N-Nylon membrane (Betagen, Waltham, MA, USA). For band visualization Kodak X-omat S film was exposed to the membrane.

2.8. PCR/RFLP analysis

To confirm a hemizygous or homozygous mutation in codon 175 of the p53 gene a PCR/RFLP analysis was applied as previously described [22].

2.9. Immunocytochemistry

Cells were grown to subconfluency and gently removed from the tissue culture dish. Slides were prepared, air-dried and fixed in methanol (−20°C) for 4 min and in acetone for 2 min followed by washes in PBS. Slides were preincubated in 5% normal horse serum and 3% bovine serum albumin diluted in PBS for 30 minutes. p53 was detected with the anti-p53 mouse monoclonal antibody PAb1801 by incubation at a concentration of 1 µg/ml for 60 min (Oncogene Science, Manhasset, NY, USA). Nonspecific mouse IgG₁ was used as negative control at the same concentration. After three washes with PBS, biotinylated horse anti-mouse IgG antibody (1:100 diluted in 3% bovine serum albumin/PBS) was added and incubated for 60 min. Antibody binding was visualized by applying the immunoperoxidase system. Slides were then dehydrated, cleared in xylene and mounted in permanent coverslipping medium. Expression of cytokeratins CK 18, CK 19 and vimentin was detected as described previously [18].

3. Results

FT-MZ-1 cells grew as a monolayer in DMEM with 10% fetal calf serum and were analyzed at passage 18. The primary tumor site was the left fallopian tube. Metastases were located in the right ovary, the broad ligament and the omentum maius. Histologically, the tumor presented as an undifferentiated papillary serous carcinoma. The cultured cells were diploid and had an S phase fraction of 24.2% with a median doubling time of 14 days. FT-MZ-1 did not form tumors after heterotransplantation of 10⁷ cells into nude mice. Cytokeratin CK 18 was detectable immunocytochemically in 95% of the cell population, CK 19 in 30% and vimentin in 98% (data not shown). High level Il-6 secretion was determined at 2051 pg/ml of the cell culture supernatant. Ovarian control cell line OV-MZ-7a showed a secretion level of only 6.6 pg secreted Il-6 per ml of the supernatant.

The entire coding region of the p53 gene was screened for mutations by heteroduplex and SSCP analysis. A heteroduplex band was observed when the PCR product comprising exon 5 was mixed with the corresponding PCR product from placental

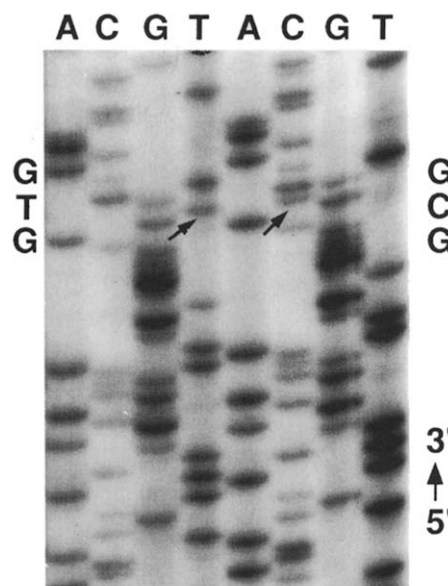


Fig. 1. Nucleotide sequence analysis of exon 5 of the p53 gene. The reactions were primed by an antisense oligonucleotide. The left panel shows the sequence of FT-MZ-1 with a second base mutation (GTG, antisense) in codon 175 (left arrow). The right panel displays the wild-type sequence of placental DNA with GCG (antisense) at codon 175 (right arrow).

DNA before renaturation (data not shown). PCR fragments from all other exons revealed only bands of expected size. In SSCP analysis, a band shift was detected in exon 5 when compared to the correspondent band pattern of the placental PCR fragment. Amplification products from all other exons did not show an altered band pattern. Sequence analysis of the exon 5 PCR product revealed a G to A point mutation (sense strand) in codon 175 altering a highly conserved arginine residue to a histidine (Fig. 1). The observed G to A transition was hemizygous or homozygous since no band indicating a G at this position was present. The mutation was confirmed in 3 independent experiments starting from genomic DNA and sequencing with a sense and an antisense oligonucleotide primer. The same results were obtained analyzing D263 and SK-BR-3 for positive control.

Since sequence analysis suggested that the detected mutation in codon 175 was hemizygous or homozygous we applied a PCR/RFLP analysis for further confirmation. A 319-bp fragment encompassing codon 175 was amplified. The *Hha*I digest of the PCR product from the fallopian tube cell line FT-MZ-1 resulted in a 67 bp band and the complete loss of the 18 bp and 48 bp bands indicating a hemizygous or homozygous alteration of the *Hha*I recognition palindrome at codon 175 (data not shown). The correspondent placental sample showed only the wild-type band pattern. Genomic DNA prepared from breast cancer cell line SK-BR-3 served as a positive control for a homozygous mutation in codon 175 [20,23].

In immunocytochemical analysis of FT-MZ-1 with PAb 1801, p53 protein accumulation was found in 75% of the cells. Marked staining was observed in the nucleus and in the cytoplasm (Fig. 2). Negative controls using nonspecific mouse IgG did not show the described staining.

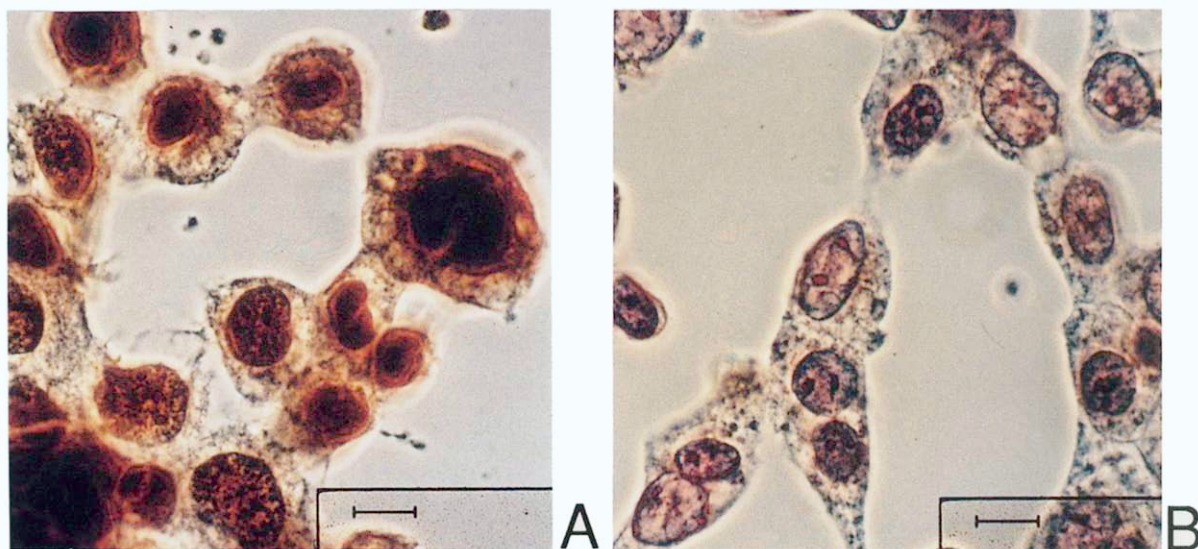


Fig. 2. Immunocytochemical analysis of p53 expression in FT-MZ-1. (A) p53-specific monoclonal antibody PAb1801. (B) Negative control. Scale marker: 10 μ m.

4. Discussion

To study molecular events in the carcinogenesis of fallopian tube cancer we have established a permanent human tumor cell line from a carcinoma of the fallopian tube. To our knowledge, no permanent cell lines of this tumor type have been reported. FT-MZ-1 was established from a solid tumor of the left fallopian tube from a 52-year-old patient at FIGO stage III who had not received any neo-adjuvant therapy prior to surgery. The tumor was histologically characterized as an undifferentiated (grading III) papillary serous carcinoma as 90% of all primary fallopian tube cancers [24]. Cytokeratin typing revealed a strong expression of cytokeratin CK 18 and CK 19 and also vimentin similar to cell lines derived from serous adenocarcinoma of the ovary [18].

Molecular analyses of the fallopian tube carcinoma have not yet been reported. Inactivating mutations of the p53 tumor suppressor gene have been described for numerous human malignancies, however not for fallopian tube cancer [4,5]. To exclude a cell culture artefact archival samples of the parental tumor of FT-MZ-1 were analyzed and found to contain the same p53 mutation (unpublished observation). The point mutation described here occurred in codon 175 located in the third of five highly conserved domains. Point mutant His¹⁷⁵ (Arg to His) has been shown to behave as an oncogenic gain-of-function mutant able to cooperate in cotransfection with activated *H-ras* in transformation of primary rat embryo fibroblasts [25]. His¹⁷⁵ exhibits less sequence-specific and less nonspecific DNA binding activity than wild-type p53 [26,27] and is unable to stimulate transcription [28]. Point missense mutations in particular codons are selected for in certain tumor types most likely for their different properties: hepatocellular carcinoma is associated with a mutational hot spot in codon 249, colorectal carcinoma in codons 175, 248 and 273 and lymphoma and leukemia in codon 175 [4,5]. Whether codon 175 is a hot spot for p53 mutations in tubal carcinoma is open to further investigation.

FT-MZ-1 cells show a constitutive Il-6 secretion at a high level. In an autocrine fashion, Il-6 appears to be implicated in

proliferation as a stimulatory factor in a number of different neoplastic cell types including ovarian cancer cells [29]. Secretion of Il-6 by the tubal carcinoma cell line FT-MZ-1 may be seen in the context of its transcriptionally inactive and homozygously present mutant p53. Expression of Il-6 can be regulated by wild-type p53 through repression of the Il-6 gene promoter [30,31]. High Il-6 expression in FT-MZ-1 may be a consequence of the loss of p53-induced transcriptional repression.

p53 His¹⁷⁵ mutant by itself neither appears to convey high proliferative potential with a doubling time of 14 days and an S phase fraction of 24.2% nor a highly tumorigenic phenotype in FT-MZ-1 cells which did not form tumors in nude mice. SK-BR-3 breast cancer cells containing the same p53 mutation [20] as well have not been found to be highly tumorigenic in nude mice [32]. Although His¹⁷⁵ is not associated with a high tumorigenic potential it may however represent a decisive step in transformation from proliferative oviduct epithelium to invasive cancer.

In conclusion, we have established a permanent human fallopian tube cancer cell line as a model to study molecular steps associated with this tumor type of which no experimental data have been reported so far. Hot spot point missense mutation His¹⁷⁵ leading to p53 protein accumulation, was identified in association with high constitutive Il-6 secretion. In fallopian tube cancer p53 analysis may become helpful in defining patients prognosis and in deciding on adjuvant therapeutic concepts.

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