Temperature-sensitive Ovarian Carcinoma Cell Line (OvBH-1)

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OvBH-1 cells from a patient with ovarian clear cell carcinoma were established and their biochemical status was analyzed. Cells grown at 37°C exhibited normal cell cycle distribution, whereas the cells shifted to 31°C were arrested in the G2/M phase of the cell cycle. Immunohistochemical analysis using anti-p53 antibodies (DO-1, PAb240, PAb421, and PAb1620) revealed that only the DO-1 antibody reacted with p53 with a high and similar percentage at both temperatures. PAb240 reacted with a low percentage of cells at 37°C and no reaction was observed at 31°C. PAb421 antibody stained a significantly lower percentage of cells at 37°C than at 31°C. Cells were not stained with PAb1620 antibody and were negative for antibodies against p21WAF1 and MDM2 proteins independently of the temperature. Sequencing of all coding exons of the p53 gene demonstrated only a neutral genetic polymorphism, i.e. a G-to-A substitution (GAG to GAA) at nucleotide position 13432. Thus, the observed temperature sensitivity of OvBH-1 cells cannot be ascribed to a p53 primary structure mutation. Based upon immunohistochemical analyses, we consider, however, that p53 in nuclei of OvBH-1 cells is in a highly unstable conformation. Furthermore, the N-terminal portion of the p53 protein at Ser20 has not been modified, and Lys373 and/or Ser378 of the C-terminus is acetylated and/or phosphorylated. The nuclear location signal of p53 is preserved. Induction of MDM2 protein is uncoupled from the cell regulatory machinery and the induction of p21WAF1 by p53 is impaired in OvBH-1 cells.

Key words: Cell line — Ovarian cancer — p53 — Temperature sensitivity — Cell cycle arrest

In order to get an insight into molecular processes which are connected with cancer, animal models and especially established cell cultures from a particular tumor type with defined properties of tumor cells are very helpful. Thus, different cell lines were established from a variety of human tumors including ovarian carcinomas. The majority of ovarian carcinoma cell lines had been established from ascitic fluid cells of patients with serous ovarian carcinoma.1–5) Only individual data exist regarding the establishment of cell lines originating from ovarian clear cell carcinomas.5–7) By characterizing a variety of human carcinomas, it turned out that p53 protein plays a key role in tumorigenesis. Mutations of p53 usually result in loss of its tumor suppression function. The p53 gene may be altered by point mutations or by extended deletions resulting in loss of p53 expression.8) Although most studies focused on identifying these mutations as factors affecting p53 function/conformation, there are several reports indicating that post-translational chemical modification may change suppressor activities of p53 in response to genotoxic stress.9) Moreover, the p53 protein can be inactivated by an altered subcellular localization in the cytoplasm or in the nucleoli10, 11) or by binding to viral or cellular proteins.12)

Reports on alterations in the p53 gene in ovarian cell lines are scarce.2, 13–17) Some authors reported point mutations in the highly conserved DNA binding domain encoded by exons 5–8 of the gene, resulting in single amino acid changes.2, 13, 15, 18) However, no data are available concerning the p53 status in ovarian clear cell carcinoma cell lines. Recently, we established a new ovarian clear cell carcinoma cell line designated OvBH-1. Its morphological and immunophenotypic characterization has been described.19) Upon extended subcultivation of the OvBH-1 cells we noticed that they were temperature-sensitive, growing normally at 37°C and eventually showing a retarded growth at lower temperature.

There are several reports of temperature-sensitive cell lines with mutations in the p53 coding sequence. A temperature-sensitive mouse p53 mutant carrying valine at amino acid position 135 instead of alanine was described by Michalovitz et al.20) Temperature-sensitive human p53 mutants with wild-type properties at 32°C and mutant properties at 37°C were also described.21–23) To shed some light on the molecular basis of the temperature sensitivity of OvBH-1 cells we decided to: i. examine their cell cycle

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distribution in response to temperature changes, ii. analyze the primary structure of p53, iii. analyze some regions of the p53 protein susceptible to post-translational modification, iv. find whether proteins induced by p53 or directing p53 to ubiquitinylation (p21WAF1 and MDM2, respectively)24, 25) are affected in OvBH-1 cells.

MATERIALS AND METHODS

Cell lines The OvBH-1 cell line was derived from the ascitic fluid cells of a patient with histopathologically diagnosed ovarian clear cell adenocarcinoma at the Department of Clinical Immunology, Medical University, Wrocław, Poland, in March 1997. The tumor was poorly differentiated and the disease stage was FIGO IV. The patient had not received chemotherapy. The morphological and phenotypic characterization and clonal homogeneity of this cell line have been described earlier.19) The epithelial origin of cells was confirmed by immunohistochemical staining using antibodies against different cytokeratin epitopes. The expression of tumor-associated antigens (CA125, OV-TL-3) reflected the origin of cells from ovarian carcinoma. In control experiments, the human cell lines MCF7,26) HT29 27) and H129928) were used.

DNA extraction DNA was extracted from the established cell line using DNAzol (Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Cell pellets were obtained by centrifugation for 10 min at 650g and 1–3×10⁷ cells were resuspended in 100 µl of phosphate-buffered saline (PBS) followed by 1 ml of DNAzol reagent. Lysis of the nuclei was performed by gentle pipetting of the mixture. After 10 min of centrifugation at 10 000g at room temperature, DNA precipitation was performed by addition of 0.5 ml of 100% ethanol. Samples were mixed by inverting tubes and were then kept at room temperature for 1–3 min. The DNA precipitate was removed by spooling with a pipette tip followed by washing twice with 0.8–1.0 ml of 95% ethanol. Subsequently, DNA samples were air-dried, suspended in 0.2–0.3 ml of AE buffer (Qiagen, Crawley, UK) and stored at −20°C.

PCR analysis The p53 gene status was studied by amplifying exons 2–11 by PCR with four sets of oligonucleotides as previously described.14) A 50 µl reaction mixture contained 100 ng genomic DNA as a template, 0.2 mM of each dNTP, 0.5 µM of each primer, 1× concentrated PCR buffer and 2.5 U Taq polymerase according to the manufacturer’s instructions (Qiagen). The amplification reaction was performed in a thermocycler (MJ Research, Watertown, MA) with an initial denaturation step of 2 min at 94°C, followed by 30 cycles consisting of three steps: 94°C for 30 s, 53°C for 30 s and 65°C for 2 min. The last cycle was followed by an extension step of 5 min at 65°C. After PCR, reaction mixtures were applied to a 2% agarose gel containing 0.4 µg/ml ethidium bromide and, following electrophoresis, fragments of appropriate length were excised. DNA from the gel fragments was extracted using NucleoTrap (Macherey-Nagel, Lennica, Poland) and subjected to either cycle or T7 sequencing. Cycle sequencing was performed using the Thermo Sequenase Cy5 Dye Terminator Sequencing Kit (APBiotech, Warszawa, Poland), with the same primers as in the PCR amplification. T7 sequencing was performed as follows: DNA was cut with EcoRI and/or AvrII (depending on the restriction sites introduced by the PCR primers) and ligated into the pLltinus vector (New England Biolabs, Beverly, MA) or directly ligated into pUC57/T (MBI Fermentas, Gда’sk, Poland) or pGEM-T (Promega, Gда’sk, Poland). Sequencing was performed using the Auto Read 200 Sequencing Kit (APBiotech) with Cy5-labeled primers according to the manufacturer’s instructions. Sequences were analyzed on an AlfExpress automated sequencer with AlfWin 2.1 software (APBiotech).

Morphology of cells OvBH-1 cells were grown at 37°C until subconfluence and then either grown further for 24 h at 37°C or shifted to 31°C for 24 h. Cytosin preparations of cells growing at both temperatures were made and fixed in cold acetone for 10 min. After staining with hematoxylin-eosin the cytomorphological features of cells grown at 37°C or 31°C were evaluated and compared.

Cytofluorometry Cells grown either at 37°C or at 31°C for 24 h were trypsinized and washed twice with ice-cold PBS. Cells were fixed and stained according to Ormerod and Kublies.300) Briefly, cells were suspended in 200 µl of PBS and incubated with 2 ml of 70% ethanol for 30 min at −20°C. Cells were centrifuged at 150g for 10 min at 4°C. The pellet was resuspended in 800 µl of PBS, 100 µl of RNase (1 mg/ml) and 100 µl of propidium iodide (400 µg/ml) and incubated for 30 min at 37°C. Cells were analyzed in a cytofluorimeter at 488 nm. Quantification of cells in the different cell cycle phases was done using Partec software (Partec, Münster, Germany).

Antibodies Immunohistochemical staining of p53 protein was carried out with the following monoclonal antibodies recognizing different epitopes of human p53: DO-1 (Novocastra, Newcastle, UK), PAb1620, PAb421 and PAb240 (obtained from University of the Saarland, Homburg, Germany). DO-1 and PAb421 recognize both wild-type and mutant forms of unphosphorylated human p53.31) In particular, DO-1 antibody detects the 20SDLWKL 25 and the PAb421 recognizes the 371SKKGQSTSRH 380 epitope localized in the C-terminus of the protein.35) The PAb1620 is generally believed to be specific for p53 in the wild-type conformation.35) Mutations or mutation-independent partial denaturation of the p53 protein destroy the PAb1620 epitope, which is not well defined and seems to

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correspond to non-sequential amino acid sequences. The PAb240 antibody detects a primary cryptic p53 epitope located between residues 213–217. Exposure of the PAb240 epitope marks at least partial denaturation of the p53 protein. All the above-mentioned antibodies were diluted 1:25. For immunohistochemical detection of MDM2 protein monoclonal antibody, clone 1B10 (NCL-MDM2) (Novocastra) at a working dilution of 1:100 was used. PAb421 protein expression was analyzed using monoclonal antibody, clone 4D10 (NCL-WAF1) (Novocastra) at 1:20 dilution. For western blot analysis PAb1620, PAb421 and DO-1 monoclonal antibodies obtained from Santa Cruz Co. (Santa Cruz, CA) were used.

**Immunohistochemical staining** Peroxidase-antiperoxidase tests were performed on cytospin preparations of OvBH-1 cells, which had been grown at 37°C and then grown further at 37°C for 24 h or shifted to 31°C for 24 h. After inhibition of endogenous peroxidase with periodic acid (2.28%) and sodium borohydride (0.02%) and 30 min of incubation with normal rabbit serum, the cell cytospin preparations were treated with primary antibodies against the p53 protein (DO-1, PAb421, PAb1620, and PAb240) or p21WAF1 or MDM2 proteins. Replacement of the primary antibodies by 0.1 M Tris-buffer, pH 7.4 served as a negative control. After 60 min incubation with primary antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) was applied for 30 min. Following washing with 0.1 M Tris-buffer, pH 7.4 the preparations were treated with peroxidase-conjugated swine anti-rabbit IgG (Dako) and the visualization was carried out with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) as a chromogen. For microscopic evaluation, the preparations were counterstained with hematoxylin and mounted. Immunohistochemical staining was evaluated by two independent observers using a double-headed BHS Olympus microscope (Olympus, Tokyo). Stained cells were determined by counting 500 cells in randomly selected fields. Cells were judged as positive if color reaction in more than 5% of cells was observed.

The cytospin preparations from ascitic fluid cells derived from patients with different histological types of ovarian carcinomas characterized by strong p53 expression (90% positive cells) were used as a positive control. As a negative control, we used cells from inflammatory fluids.

**Immunofluorescence** Cells were grown on coverslips until they were 50–70% confluent, then fixed in 2% formaldehyde in PBS, pH 7.4 for 15 min at 20°C, washed with PBS for 3×10 min, and permeabilized with 0.2% Triton X-100 containing 1% normal goat serum for 5 min on ice. Cells were washed again and then incubated with PAb421, DO-1 or PAb1620 antibody at the appropriate concentration for 1 h at room temperature in a humidified chamber. Cells were washed with PBS for 3×10 min and then incubated with the FITC-conjugated goat anti-mouse antibody at room temperature for 1 h in a humidified chamber. Finally, the cells were washed again with PBS (4×10 min). The coverslips were fixed with a drop of mounting medium and analyzed under a fluorescence microscope.

**Western blot analysis** Cells grown at 37°C for 24 h or shifted to 31°C for 24 h were extracted with extraction buffer (50 mM Tris-HCl, pH 9.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP40) for 30 min on ice. The extract was centrifuged for 30 min at 16 000 g at 4°C, and 120 µg total protein was loaded on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics, Mannheim, Germany) and the filter was incubated either with DO-1, PAb421 or PAb1620 antibody followed by anti-mouse IgG-POD (Dianova, Hamburg, Germany). Proteins were detected with the enhanced chemiluminescence (ECL) system (Roche Diagnostics).
RESULTS

Upon subculturing of the OvBH-1 cells, a reduced growth was observed at temperatures lower than 37°C. Thus, the morphology of OvBH-1 cells grown at 37°C and either kept at 37°C or shifted to 31°C was analyzed (Fig. 1, a and b). It appeared that, independently of temperature, the cells showed similar cytomorphological features of malignancy. At both temperatures the OvBH-1 cells were large in size with indistinct cell borders. The cytoplasm contained small vacuoles and the nuclei were pleomorphic with central or atopic localization, showing variations in size and shape. In the next step, the cell cycle distribution of OvBH-1 cells was investigated more systematically. Cells were grown at 37°C and then either cultured further at 37°C or shifted to 31°C for 24 h. Cells were fixed, stained with propidium iodide and analyzed by cytofluorimetry. As shown in Fig. 2 a cells grown at 37°C were mainly in the G1 phase of the cell cycle (48.80%), and minor fractions were in the S (17.60%) or G2 phase (20.41%). After 24 h at 31°C, most of the cells were in G2 (32.83%), and some in S (20.96%) and G1 (27.55%), indicating that cells might have gone into G2/M arrest (Fig. 2b). Shifting the cells back to 37°C for another 24 h led to a cell cycle distribution which was very similar to the one shown in Fig. 2a (data not shown). In control experiments, MCF7 cells containing wild-type p53, HT29 cells

![Fig. 2. Cell cycle analysis of OvBH-1 line cells grown at 37°C (a) and shifted to 31°C (b).](image)

![Fig. 3. Reactivity of DO-1 antibody with OvBH-1 cells grown at 37°C (a) and 31°C (b) and PAb421 with cells grown at 37°C (c); immunoperoxidase staining, 40 μm scale bar for a, b, c.](image)
expressing p53 mutant with codon 273 resulting in an Arg→His substitution, and H1299 cells which do not contain p53 were analyzed. None of these cell lines showed a G_{2}/M arrest, as OvBH-1 cells do (not shown).

It is known that temperature sensitivity of cell growth might be due to mutated p53, which is characterized by conformational changes that prolong the half life and stability of the protein and which enable immunohistochemical detection of p53. As demonstrated in Fig. 3, a and b OvBH-1 cells exhibited very strong nuclear overexpression of p53 at 37°C and at 31°C as judged by DO-1 antibody staining. Monoclonal antibody PAb421 stained a significantly lower percentage of cells ranging from 5% at 37°C to 20% at 31°C (Fig. 3c). The cells were not stained

![Fig. 4. Immunofluorescence staining with DO-1 (a, b) and PAb421 (c, d) antibodies of cells of OvBH-1 cell line grown at 37°C and at 31°C. For comparison phase-contrast (Ph) pictures of cells are also presented; 40 µm scale bar for a, b, c, d.](image-url)
with PAb240 antibody at 31°C, whereas at 37°C in some passages the number of stained cells varied within the range of 0–60% (mean value 25%) (not shown). At either temperature, no reactivity with monoclonal antibody PAb1620 was found.

In the next step we performed an immunofluorescence analysis of OvBH-1 cells with DO-1, PAb421 and PAb1620 antibodies. As shown in Fig. 4, a and b, a strong nuclear staining with DO-1 and a very weak reactivity with PAb421 (Fig. 4, c and d) were observed. In all cases, nucleoli as well as the cytoplasm were negative for p53. No immunostaining was observed with PAb1620 (data not shown).

In order to confirm the results described above by biochemical methods, we repeated the temperature shift experiments. Cells were extracted and proteins were analyzed on an SDS polyacrylamide gel followed by western blotting with three monoclonal antibodies—DO-1, PAb421, and PAb1620. As shown in Fig. 5a, only the DO-1 antibody detected a protein band corresponding to p53, whereas PAb421 and PAb1620 were both negative. Moreover, there was no substantial difference in the p53 signal when the same amount of total protein extracted from cells grown at 37°C or at 31°C was assayed (Fig. 5a). Thus, the biochemical analyses are basically in agreement with the results obtained with immunohistochemistry and also immunofluorescence (see “Discussion”).

In independent experiments, proteins associated with function of p53, namely MDM2, which may direct p53 to degradation,24) and p21WAF1, which can be induced by p53 in response to DNA damage and may direct cell cycle arrest in G1/S phase,25) were also analyzed. It was found that, independently of the temperature, cells were negative for both p21WAF1 and MDM2 proteins (data not shown).

Taking into account that temperature sensitivity of cell growth might be due to mutation of p5320–23) we looked for the possible p53 gene abnormalities. This gene contains eleven exons including the first noncoding exon.40) Therefore, the template DNA that forms the OvBH-1 cells was PCR-amplified for exons 2–11 and sequenced. Surprisingly, we found no mutation in this region which would lead to changes in the primary structure of the p53 protein. However, we observed a neutral genetic polymorphism, namely a G-to-A substitution at nucleotide 13 432 GAG to GAA, corresponding to codon 224 in exon 6 (Fig. 5b). It is reasonable to conclude that the temperature-sensitive growth of OvBH-1 cells does not originate from changes in the primary structure of p53. Thus, although most temperature-sensitive lines are dependent on p53 mutation, the OvBH-1 cell line is not.

**DISCUSSION**

We have previously described the establishment and morphological and phenotypic features of the OvBH-1 cell line.49) Upon culturing these cells, it turned out that they have very interesting features. First of all, the cell line shows temperature-sensitive growth characteristics. Cells grow well at 37°C and they stop growing when shifted to 31°C. Cell cycle analysis revealed that most of the cells were arrested in the G2 phase. A transient G2/M block was recently described for ovarian carcinoma cell lines after exposure to cisplatin and taxol.13 41–43) These drugs induced cell cycle arrest and allowed DNA repair in the G2 phase prior to mitosis. Depending on the success of repair, cells may emerge from the G2 phase and re-enter the normal cell cycle. If DNA repair fails, cells may enter the apoptotic pathway.43) Evaluating the role of p53 and p21WAF1 in senescence-like terminal differentiation arrest induced in human tumor cell lines by chemotherapy, Chang et al.44) demonstrated that these proteins act as positive regulators of proliferation arrest. Their function, however, seems to be not necessary for this kind of response to anticancer drugs. It is well established that p21WAF1 induction results in G1/S arrest of the cell cycle.45 46) Moreover, p21WAF1 is also required for arrest in the G2 phase of the cell cycle. Since p21WAF1 is known to bind to proliferating cell nuclear antigen (PCNA) there is some indication that p21WAF1 might regulate G2 arrest by blocking the interaction between cdc25C and PCNA.47) Our finding that OvBH-1 cells are negative for p21WAF1 may indicate that...
the $G_2$ arrest in OvBH-1 cells is $p21^{WAF1}$-independent. However, OvBH-1 cells exhibit complex karyotype characteristics with clearly visible aberration of nearly all chromosomes (unpublished results), indicating that a number of other genes might be altered in these cells.

It has been demonstrated previously that temperature-sensitive mutant p53 causes the arrest of cells in the $G_2/M$ phase of the cell cycle. This arrest goes along with an increase of the amount of p53 after shift of the cells from 37°C to 32°C. However, our immunohistochemical as well as the western blot experiments showed comparable amounts of p53 recognized by DO-1 antibody in cells grown at 37°C and those shifted to 31°C. Also, morphological features of the cells appear to be temperature-independent. Moreover, sequence analysis revealed that there is no p53 mutation in the region expected to be involved in the generation of a temperature-sensitive p53. On the other hand, a silent mutation at codon 224 in exon 6 was found, which is unlikely to contribute to temperature sensitivity. A remarkable feature of the OvBH-1 cells, however, is the elevated level of p53 protein which strongly reacts only with one (DO-1) out of four different monoclonal antibodies tested. In immunoperoxidase and immunofluorescence studies, only a weak staining of cells with PAb421 was observed, whereas in the western blot analysis the same antibody did not react with p53. It seems that p53 is in a very labile conformation in OvBH-1 cells and is unable to refold to “semi-native” structure after protein extraction and SDS treatment preceding the western blot analysis.

Analysis of p53 levels in various cell types has shown that several genotoxic factors stabilize p53 and induce its accumulation in the nucleus. Stabilization is thought to result primarily from disruption of the interaction between p53 and MDM2, which targets p53 for ubiquitin-mediated degradation. It has been demonstrated that phosphorylation of Ser20 mediates stabilization of p53 in response to DNA damage. It is known that the last 30 amino acid residues of the C-terminal domain of p53 negatively regulate the specific DNA binding by the p53 central core domain. Post-translational modification may relieve this inhibition mediated by the C-terminus. For example, acetylation, which may also be important for suppression of oncogenic ras-induced transformation, stimulates sequence-specific DNA binding. Recent results show that MDM2 suppresses p300/CBP-mediated p53 acetylation. Decreased interaction of PAb421 with p53 in OvBH-1 cells at 37°C, in comparison to the cells shifted to 31°C, indicates that at higher temperature, p53 is modified in the C-terminus, possibly by acetylation at Lys373 in response to greater stress. However, this decreased immunoreactivity may also be caused by phosphorylation at Ser378 and this would indicate that more cells are in a quiescent phase. At present, we cannot discriminate between those two possible sites of p53 modification.

It is generally believed that changes in the p53 tertiary structure can be defined by a series of monoclonal antibodies which recognize different conformational states of this protein. One of them is the PAb1620 antibody, which recognizes a non-linear epitope only in the p53 protein having wild-type conformation. The presence of a specific mutation or the partial denaturation of the p53 protein destroys the PAb1620 epitope. Our observation that PAb1620 was unable to recognize the p53 protein and the fact that we found no mutation in the p53 gene suggest that the p53 protein in OvBH-1 cells is in an at least partially denatured state. This is further supported by our preliminary finding that about 40% of OvBH-1 cells are stained with PAb240 antibody at 37°C, whereas at 31°C the cells remained negative (unpublished data). It appears that the cryptic epitope between amino acids 213 and 217 becomes exposed in p53 at higher temperatures in OvBH-1 cells. Such exposure is characteristic of transition of the p53 structure to a denatured state.

Nuclear accumulation of p53 may result from imbalance in nucleoplasmic shuttling. It has recently been shown that p53 shuttles between the nucleus and cytoplasm through its intrinsic nuclear localization signal and nuclear export signal. Although the mechanism of this transport is complex and depends on the conformation of p53 and other proteins, it is conceivable that the nuclear localization signal in the p53 tetramerization domain is functioning in the OvBH-1 cells.

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human tumor model for investigation of cell cycle regulators and p53 structure status.

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