Introduction

The HMGA family is composed of three proteins: HMGA1a, HMGA1b and HMGA2. The first two proteins are products of the gene HMGA1, generated through an alternative splicing mechanism (1). HMGA proteins are involved in the regulation of chromatin structure, and HMGA-DNA-binding sites have been identified in the functional regions of many gene promoters (2). HMGA1 proteins also have been shown to bind to other transcription factors, such as NFkB, Elf-1 and Tst-1/Oct-6, through protein–protein interactions occurring in highly conserved regions such as Ets domain of Elf-1 and POU domains of Tst-1/Oct-6. Thus, HMGA1 proteins are considered able to induce transcription (3–5).

HMGA genes, expressed at absent or low levels in normal adult tissues, may have an important role in the development and regulation of adipogenesis. In fact, they are abundantly expressed during embryogenesis (6,7) and mice carrying a disrupted HMGA2 gene show a pygmy phenotype with a great reduction in fat tissues (6). Conversely, mice carrying an activated HMGA2 gene are giant, with diffuse lipomatosis (8,9). Recent work also shows that the HMGA1 proteins play a critical role in adipocytic cell growth and differentiation (10).

Over-expression of the HMGA1 gene was first demonstrated in rat thyroid cell malignantly transformed by several oncogenes (11–13). A high HMGA1 expression also has been reported in several human neoplasias, including thyroid (14,15), prostate (16), cervix (17), colorectum (18–20) and pancreatic carcinomas (21), suggesting that HMGA1 over-expression is a general feature of the malignant phenotype and is independent of the cell type undergoing transformation. This hypothesis seems supported by several findings demonstrating that over-expression of the HMGA family of proteins is causally associated with both neoplastic transformation and metastatic progression, and summarized as follows. (i) The block of HMGA2 protein synthesis prevents rat thyroid cell transformation by murine transforming retroviruses (22). (ii) An adenovirus carrying the HMGA1 gene in antisense orientation induces cell death in thyroid anaplastic, breast and lung carcinoma cell lines (23). (iii) Increased expression of HMGA1 or HMGA2 leads to transformation with anchorage-independent cell growth in two experimental cell lines (24) and Rat la cells over-expressing HMGA1 or HMGA2 form tumors in nude mice with the presence of distant metastases (25). (iv) Human breast epithelial cells harboring a tetracycline-regulated HMGA1 transgene acquire the ability to form both primary and metastatic tumors in nude mice only when the transgene is actively expressed (26). (v) Expression of either antisense or dominant-negative HMGA1 constructs inhibits both the rate of proliferation of tumor cells and their ability to grow anchorage independently in soft agar (26); and, finally, transgenic mice over-expressing the wild-type or a truncated HMGA2 gene develop natural killer cell lymphomas (27).

Abbreviations: GAPDH, glyceraldehyde-phosphate-dehydrogenase; HMGA1, high mobility group A1; LMP, low malignant potential.
The ovary is the fifth most frequent site of cancer among western women, and ovarian cancer is the fourth leading cause of cancer death (28). Among patients with gynaecological cancer, more deaths occur from ovarian cancer than from cervical and endometrial cancers combined. Although understanding of the molecular events underlying the pathogenesis of this tumor has improved, additional studies are needed because characterization of the genetic alterations occurring in ovarian carcinomas may improve the diagnosis and suggest novel therapeutic approaches. For this purpose, 44 epithelial ovarian specimens were investigated for HMGA1 expression by immunohistochemistry. HMGA1 proteins were not detected in normal ovarian surface epithelium, but were detected at low levels in some low malignant potential (LMP) tumors. HMGA1 proteins were abundantly expressed in most of the primary ovarian adenocarcinomas investigated. Results from western blot analysis and RT–PCR were consistent with immunohistochemical data. In addition, we demonstrated growth arrest of the human ovarian cancer cell lines OVCAR-5 and OVCAR-8 by an adenovirus carrying the HMGA1 gene in an antisense orientation (Ad-Yas-GFP). These results support a role for HMGA1 over-expression in human carcinogenesis and suggest that HMGA1 could be an important target for gene therapy of ovarian cancer.

Materials and methods

Cell lines

The human ovarian carcinoma cell lines CAOV-3, CAOV-4, ES-2, TOV-112D, TOV-21G, MDAH-2774, SW-626 and OV-90 were obtained from the American Type Culture Collection (Manassas, VA). The OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SKOV-3 and IGROV were kindly provided by Dr Gabriela Tudor (National Cancer Institute, Frederick, MD). A2780, whole ovary and normal ovarian surface epithelium cell lines were a gift from Dr Andrew Godwin (Fox Chase Cancer Center, Philadelphia, PA). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 (Gibco, In Vitrogen, Carlsbad, CA) containing 10% fetal calf serum, 100 μg/ml streptomycin and 100 IU/ml penicillin.

Tumor samples

Forty-four fresh ovarian specimens were obtained from patients who underwent surgical resection at the “Istituto Nazionale dei Tumori”, Naples, Italy and at the Catholic University of Rome, Italy. Twenty-nine specimens were defined as primary malignant tumors, whereas 10 specimens were classified as LMP tumors, one as metastatic ovarian adenocarcinoma and four as normal ovarian tissue. Histological classification of tumors was carried out according to the WHO system, and tumors were graded as well (G1), moderately (G2) and poorly differentiated (G3). Clinical stages of disease were established according to the FIGO staging system. After surgical resection, each tumor specimen was divided into two portions; one portion was instantly frozen in liquid nitrogen for protein and RNA extraction, while the second portion was formalin-fixed and paraffin-embedded for histologic and immunohistochemical investigation.

Immunohistochemical analysis of tissue samples

For immunohistochemistry, 5–6 μm paraffin sections were deparaffinized, followed by quenching of endogenous peroxidase activity by 0.3% hydrogen peroxide in methanol. After rinsing with PBS, the sections were incubated with normal serum as the blocking reagent, and then incubated with primary antibody anti-HMGA1 antibody at a dilution 1:100 for 14 h at 4°C. This antibody was raised against a HMGA1-specific synthetic peptide corresponding with the N-terminal region of the molecule (14). The slides were then incubated with biotinylated anti-rabbit IgG for 30 min and, after washing in PBS, with avidin–biotin–peroxidase complex for 30 min. Diaminobenzidine (Vector, Burlingame, CA) was used as the final chromogen, and Mayer’s hematoxylin was used as a counterstain. For the negative control, PBS was substituted for the primary antibody. The specificity of the reaction was confirmed by lack of tissue immunoreactivity after pre-incubation of the antibody with molar excess of the HMGA1 synthetic peptide (pre-absorption test). Two pathologists (J. Palazzo and G. Chiappetta) separately evaluated HMGA1 staining. At least 20 high-power fields were chosen randomly and 1000 cells were counted. Tissue samples were scored as positive in the presence of ≥10% of cancer cells with positive nuclear staining.

RNA extraction and northern blot analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Northern blot hybridization was performed according to standard procedures (29) using Hybond-XL membranes (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The membranes were hybridized with the HMGA1 probe (1) radiolabeled with the Prime-It III random primer labeling kit (Stratagene, La Jolla, CA). Hybridization reaction was performed at 42°C with a working concentration of the probe of 2 × 106 c.p.m./ml in hybridization mix. After washes, filters were air-dried and exposed to autoradiographic film for 1–4 days. RNA was used to ascertain equal RNA loading.

Immunoblot analysis of ovarian cancer cell lines and tissue samples

Total proteins were extracted from normal and neoplastic tissues or cell lines following standard techniques as described previously (7). The extracts separated by 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to Immobilon-P Transfer membranes (Millipore, Bedford, MA). After staining with S Ponceau for normalization, membranes were blocked with 5% non-fat milk proteins and were incubated O.N. with a polyclonal antibody raised against the N-terminal region of the HMGA1 protein at a dilution of 1:1000 (14,15). As a loading control, β-actin (sc-8432) and β-tubulin (sc-5274, Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies were used at a dilution of 1:1000. Bound antibodies were detected by appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Pharmacia Biotech AB).

RT–PCR analysis of the HMGA1 gene expression

Total cellular RNA was extracted from frozen tissues and cell lines in log phase growth using TRI Reagent (Sigma, St Louis, MO). Two micrograms of DNase-treated total RNA were reverse transcribed using the random hexamers cDNA synthesis kit (Promega, Madison, WI). Subsequently, 1 μl of cDNA was used for each PCR reaction with each primer pair. PCR reactions were performed in a final volume of 100 μl containing the following: 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.5 μM of each primer and 2.5 U Tag DNA Polymerase (Promega). The PCR reaction for HMGA1 was performed for 30 cycles (94°C for 30 s, 55°C for 2 min and 72°C for 2 min). The following primers were used: 5'-GGCACTGAGAAGCGGGGCCG-3' and 5'-CCCTTGTGTTTTTGCTTCCCTT-3' (corresponding to the nucleotides 68–88 and nucleotides 141–161, respectively, of HMGA1 cDNA). In addition, a set of primers specific for the constitutively expressed enzyme glyceraldehyde-phosphate-dehydrogenase (GAPDH) was added to each reaction after 20 cycles of PCR to serve as an internal control for the amount of cDNA tested. The GAPDH specific primers were forward 5'-ACATGTTCCAAATAT- GATTCC-3' corresponding to the nucleotides 194–214, reverse primer: 5'-TGGACTTCCACGAGCTACTCAG-3' corresponding to the 336–356 nucleotides. PCR products were visualized after electrophoresis on a 2% agarose gel, and then transferred to GeneScreen plus nylon membrane (Dupont, Boston, MA) and hybridized with a HMGA1 probe (14,15). Integrated absorbance values were obtained using a PhosphorImager (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA) and analyzed by the ImageQuant gel analysis software. The relative level of HMGA1 expression was assessed by comparison with the level of GAPDH in the same sample.

In vitro transduction

Ad-GFP and Ad-Yas-GFP were generated as described previously (23). Ovarian cancer cells (5 × 106) were seeded in a six-well system in a medium containing 10% calf serum. After 24 h, cells were exposed to 10 and 100 p.f.u./cell of Ad-GFP or Ad-Yas-GFP. After a 24-h incubation at 37°C, the virus was removed and the incubation continued in fresh medium at 37°C. Cells were trypsinized each day and counted by using a hemocytometer. The transduction efficiency was determined by measuring the proportion of GFP expressing cells after Ad-GFP transduction by fluorescence-activated cell sorting (FACS) (Becton Dickinson, San Jose, CA). Direct visualization of the Ad-GFP-transduced cell population by fluorescent cell microscopy was used to confirm the FACS data.

Results

HMGA1 expression in ovarian carcinoma cell lines

We analyzed the expression of the HMGA1 protein by western blot in a series of ovarian carcinoma cell lines and primary ovarian adenocarcinomas. All specimens showed a band of
15 kDa corresponding to the HMGA1 protein (Figure 1A and B) and with the OV-90, MDAH-2774, OVCAR-5, SW-626 and ES-2 cell lines expressing the highest levels of HMGA1. Conversely, no HMGA1 expression was present in normal ovarian surface epithelium and only a faint HMGA1 band was detectable in the whole normal ovary (Figure 1A and B). To verify that the same amount of total protein was present in each lysate, the same blots were reprobed with a monoclonal antibody against β-actin or tubulin (Figure 1A and B).

Northern blot analysis was performed on the same panel of ovarian cancer cell lines to determine if the HMGA1 expression correlated with mRNA level. Figure 1C shows that a HMGA1-specific transcript is detectable in all of the ovarian carcinoma cell lines but is considerably weaker in the whole normal ovary, suggesting a transcriptional activation of the HMGA1 gene in ovarian cancer. However, lack of correlation between mRNA and HMGA1 protein levels in some cell lines suggests that post-transcriptional mechanisms, as acetylation and/or phosphorylation, also might play a role in the regulation of HMGA1 expression in ovarian cancer.

Immunohistochemical detection of HMGA1 proteins in ovarian neoplastic tissues

Forty ovarian neoplastic samples, including 29 primary invasive carcinomas, one metastatic ovarian adenocarcinoma, 10 LMP tumors and four normal ovarian tissues were analyzed by an immunohistochemical assay using an antibody raised versus the N-terminal region of the HMGA1 protein (14). This antibody is not able to detect the HMGA2 gene, the product of the other member of the HMGA family. The results are summarized in Table I. Consistently with western blot data, no HMGA1 expression was detected in any of the four samples of normal ovarian surface epithelium (Figure 2A), which is the area from which epithelial tumors arise. Conversely, immunoreactivity for HMGA1 was found in some LMP tumors and in most of the primary invasive carcinomas (Table I). LMP tumors exhibited a weak focal immunostaining for HMGA1 (Figure 2C) and were scored as positive in four out of 10 samples (40%). As expected, immunohistochemical reactivity was predominantly localized to the cell nuclei; however, some cytoplasmic staining was also observed. Figure 2B and D shows a representative LMP negative and positive sample.

Primary ovarian adenocarcinomas expressed HMGA1 in 19 out of 30 samples (63%) (Figure 2E), whereas absence of HMGA1 protein expression was observed in the remaining samples (37%) (Figure 2G). The majority of these tumors showed a very intense nuclear staining (Figure 2E). In most samples cytoplasmic staining also was observed (Figure 2E and H) as already reported for thyroid and colon neoplasias (15,18). The high levels of HMGA1 proteins present in the samples may account for the latter result. An intense nuclear and a diffuse cytoplasmic staining also was consistently observed in a metastatic ovarian sample (Figure 2F). As shown in Table I, the percentage of HMGA1-positive cells in the tumor was >80% in half of the samples analyzed, which

<table>
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<th>Histological type of ovarian specimens</th>
<th>No. of positive cases/no. of cases analysed</th>
<th>Positivity (no. of cases)</th>
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<td></td>
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<tr>
<td>Normal ovarian surface epithelium (4)</td>
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<td>LMP tumors (10)</td>
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<td>6/10</td>
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<tr>
<td>Primary ovarian carcinomas (30)</td>
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<td>11/30</td>
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P = n.s.

a70–90% of cells are not immunostained, the remaining cells show faint immunostaining.

b50% of cells show no immunostaining or faint immunostaining, whereas the remaining 50% show intermediate or strong immunostaining.

cAt least 80% of cells show intermediate or strong immunostaining.
Fig. 2. HMG1 immunostaining in normal ovarian surface epithelium, LMP ovarian tumors, primary and metastatic ovarian adenocarcinomas. (A) No immunoreactivity was observed in normal ovarian surface epithelium (magnification ×250). (B–D) HMG1 immunostaining in two LMP tumors. HMG1 is not expressed in one sample (B), in contrast a weak and focal staining is seen in the other specimen (C). HMG1 is mostly nuclear; however, some cytoplasmic staining is also present (D). Magnification ×150 (C) and ×250 (B and D). (E, G and H), HMG1 immunostaining in three representative samples of primary ovarian adenocarcinomas. The majority of PICs showed a very intense nuclear staining (E) and some cytoplasmic staining (E and H); however, in some samples no HMG1 immunostaining was detectable (G). Magnification ×250. (F) An intense nuclear and a diffuse cytoplasmic staining also was observed in a metastatic ovarian sample. Magnification ×250.
indicates that the vast majority of carcinoma cells expressed high levels of HMGA1 protein. The specificity of the reaction was validated by the absence of staining when carcinoma samples were stained with antibodies pre-incubated with the peptide against which antibodies were raised. Similarly, no staining was observed in the absence of the primary antibodies (data not shown).

**RT–PCR analysis of the HMGA1 gene expression**

To validate the immunohistochemical data, we investigated by semi-quantitative RT–PCR analysis whether similar HMGA1 expression was present in a subset of 40 representative ovarian samples. mRNA was isolated from frozen tissues, subjected to reverse transcription, and analyzed by PCR and the results are reported in Table II. Figure 3 shows some representative samples of ovarian carcinoma over-expressing HMGA1. Consistently with northern and western blot data, weak HMGA1 expression was detected in normal ovary (Figure 3). The majority of the primary invasive carcinomas (65%) showed HMGA1 expression, whereas only 35% of the LMP tumor samples expressed this protein (Table II). These results correlated highly with the immunohistochemical findings \((P = 0.004)\). In addition, the highest HMGA1 mRNA levels were detectable in primary ovarian adenocarcinomas as compared with LMP tumors (Figure 3), thus supporting the hypothesis that levels of HMGA1 expression are related directly to tumor aggressiveness. As a control, the same reverse transcription products were co-amplified with primers targeted to GAPDH, and the product was identified in all samples (Figure 3).

### Table II. HMGA1 gene expression in human ovarian tissues analyzed by RT–PCR

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<tr>
<th>Histological type of ovarian specimens (no. of cases)</th>
<th>No. of positive cases/no. of cases analyzed</th>
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<td>Normal ovarian surface epithelium (2)</td>
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<tr>
<td>LMP tumors (14)</td>
<td>5/14</td>
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<td>Primary ovarian carcinomas (23)</td>
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\(P = \text{n.s.}\)

**Infection of ovarian carcinoma cell lines by Ad-Yas-GFP suppresses HMGA1 protein synthesis and cell growth**

We showed recently that over-expression of the HMGA1 proteins is associated with the malignant phenotype of human carcinoma cells, because an adenovirus carrying the HMGA1 gene in antisense orientation (Ad-Yas) induced cell death in two thyroid anaplastic carcinoma cell lines, but not in normal thyroid cells (23). We therefore investigated the effects of the suppression of HMGA1 protein synthesis on the cell growth of two ovarian carcinoma cell lines, OVCR-5 and OVCR-8. For this experiment, we used a variant of the Ad-Yas virus, already described (21), carrying the GFP gene. We first demonstrated, by direct fluorescence, that the virus was able to infect these cells (data not shown). Subsequently, western blot analysis showed a significant reduction of HMGA1 protein levels in both OVCR-5 and OVCR-8 4 days after the infection with 10 and 100 p.f.u./cell of Ad-Yas-GFP (Figure 4B and D, lanes 3 and 4). Conversely, when both of these cell lines were uninfected (Figure 4B and D, lane 1) or were infected with 100 p.f.u./cell of Ad-GFP (Figure 4B and D, lane 2) only a slight reduction in HMGA1 protein synthesis was shown. No change in GRB-2 protein levels was observed in the two cell lines infected with the same adenoviruses, which indicates that equal amounts of proteins were loaded and that no non-specific inhibition of protein synthesis occurred in the infected cells.

To study the effect of Ad-Yas-GFP on ovarian carcinoma cell growth, we plated OVCR-5 and OVCR-8 cells (50,000/well), and we exposed them to 10 and 100 p.f.u./cell of Ad-Yas-GFP or Ad-GFP the following day. The treated cells were harvested daily for cell counting. As shown in Figure 4A and C, infection of both cell lines with Ad-Yas-GFP virus drastically reduced the cell number over the 4 days of examination. Conversely, when both OVCR-5 and OVCR-8 were infected with 10 and 100 p.f.u./cell of Ad-GFP, cells showed only a slight decrease in their growth rate. Similar results were also obtained by infecting the IGROV-5 carcinoma cell line with the Ad-Yas-GFP virus (data not shown). These results suggest that gene therapy based on the
suppression of HMGA1 protein function could be considered for the treatment of ovarian carcinomas.

Discussion

Ovarian cancer is the most frequent fatal cancer of the female reproductive tract in industrialized countries. It is the fourth leading cause of cancer death among women of Western countries after breast, lung and colorectal cancer, and has a lifetime risk of ~2% (28,30,31). Despite progress in surgical and chemotherapy treatment, the 5-year survival rate for all stages of ovarian cancer has remained constant at 30% over the past 30 years.

At present little is known about molecular etiology of ovarian malignancies. Several reports show that most ovarian cancer occurs as a result of acquired alterations in oncogenes and in tumor suppressor genes involved with the regulation of cell proliferation and differentiation, as well as with cell-cycle control (32,33). Studies have shown that mutations in the BRCA1 and BRCA2 genes are frequently present in Human Ovarian Cancer (HOC) families, whereas few somatic mutations have been detected in sporadic ovarian cancer patients (34–36). Other reports also suggest a role for aberrations of chromosome 6, mainly involving deletions from 6q (37–39).

An increase of PIK3CA copy number in ovarian cancers has also been reported, and treatment with the PI3-kinase inhibitor LY294002 was able to decrease proliferation and increase apoptosis in ovarian cancer cells (40). A high incidence of PTEN mutations and 10q23 loss of heterozygosity recently was reported in patients with synchronous endometrial and ovarian carcinomas (41). Specifically, inactivation of the PTEN tumor suppressor gene appears to be an early event in the development of ovarian endometrioid carcinoma and of clear cell carcinoma of the ovary (42).

A better understanding of the genetic events involved in the pathogenesis of ovarian carcinomas may help in both the diagnosis and treatment of this malignancy.

In our study we analyzed the expression of the HMGA1 proteins in a panel of ovarian carcinoma cell lines and tissues. The rationale for this investigation was the detection of the HMGA1 proteins in several human carcinomas (14–21) and the report of a possible prospect of a cancer treatment based on the suppression of the HMGA1 protein synthesis (23).

We show here that the induction of the HMGA1 proteins is a frequent event in ovarian tumors, particularly in ovarian adenocarcinomas and is mostly transcriptionally regulated. However, a subset of tumors do not show the induction of the HMGA1 gene. This result appears to contrast with previous
results showing the expression of the HMGA1 in all the malignant thyroid, colonic and pancreatic neoplasias. Some hypotheses might be envisioned to account for this discrepancy: (i) different molecular events might be involved in ovarian carcinomas. It also cannot be excluded that some genetic alterations leading to cancer do not involve HMGA1 gene expression and (ii) other HMGA-related genes may be induced in the process of ovarian carcinogenesis.

In this study we also report a reduced expression of HMGA1 in LMP ovarian carcinomas as compared with primary ovarian carcinomas. This result is consistent with previous reports with thyroid, colon and cervix neoplasias, where the HMGA1 protein levels correlate with the malignant phenotype of these tumors (17,20,21). HMGA1 staining, then, could provide an additional molecular distinction between these two major categories of epithelial ovarian cancers.

Ad-Yas-GFP suppresses HMGA1 protein synthesis and drastically reduces the proliferation rate of OVCAR-5 and OVCAR-8 human ovarian carcinoma cell lines, and suggests that a therapy based on the block of HMGA1 could be effective in the inhibition of ovarian cancer cell growth.

Over-expression of the HMGA1 proteins might have a specific role in the pathogenesis of ovarian carcinomas. In fact we demonstrate that HMGA1 is able to negatively regulate the expression of the BRCA1 gene, which is mutated in most of the familiar ovarian carcinomas, and shows a reduced expression in the sporadic forms (43). The induction of HMGA1 expression in ovarian carcinogenesis down-regulates BRCA1 gene expression, probably accounting for their aggressive phenotype. Consistent preliminary results obtained in our laboratory indicate that an inverse correlation exists between HMGA1 and BRCA1 protein levels in a panel of human ovarian carcinomas (manuscript in preparation).

The abundant expression of HMGA1 proteins in primary and metastatic ovarian carcinomas compared with LMP tumors, points to a potential role for HMGA1 in distinguishing between tumors with different biological behavior. Our results also suggest that a gene therapy based on the suppression of HMGA1 protein function could represent a new and promising approach for the treatment of ovarian cancer.

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References


