hMSH2 and GTBP expression in advanced stage epithelial ovarian cancer

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Summary Defects in DNA mismatch repair have been associated with both hereditary and sporadic forms of human cancer. Most of the attention has been focused on the incidence and genetics of mismatch repair defects, while little is known about the expression levels of the mismatch repair proteins and their significance in cancer cell biology. In this study, both the expression levels of hMSH2 and GTBP proteins were investigated by Western blotting in 20 untreated epithelial ovarian cancers. For these analyses, a commercial anti-hMSH2 monoclonal antibody and a newly generated mouse monoclonal anti-GTBP antibody were used. hMSH2 and GTBP proteins were detected by Western blotting in 19 out of 20 (95%) samples analysed and were found to be directly correlated ($r = +0.51$, $P = 0.025$). hMSH2 expression was significantly higher in ovarian cancer cells originating from solid tumours than from ascites ($H = 4.5$, $P = 0.033$), whereas GTBP content did not significantly differ according to the origin of cancer cells. No statistically significant differences were found in the distribution of hMSH2 and GTBP levels according to the age of the patients, grade of differentiation, histotype and extent of surgical debulking. The amount of hMSH2 protein was demonstrated to be significantly lower in stage IV than in stage III patients ($H = 7.35$, $P = 0.007$). Moreover, significantly lower hMSH2 levels were observed in non-responding patients compared to patients who achieved complete or partial response to cisplatin-based chemotherapy ($H = 4.88$, $P = 0.027$). Conversely, GTBP levels were not distributed differently according to stage of disease and chemotherapy response. Our study suggests a possible involvement of hMSH2 in ovarian cancer cell biology and susceptibility to chemotherapy. The possible biological and/or clinical role of GTBP expression in ovarian cancer patients remains to be elucidated.

Key words: hMSH2; GTBP; ovarian cancer; cisplatin
some human colorectal cell lines deficient in hMLH1 or hMSH2 are more resistant to CP than the sublines in which the MMR defect has been complemented by chromosome transfer. Finally, Brown et al (1997) suggested that loss of hMLH1 expression may be critically involved in the development of CP resistance in ovarian cancer patients. Although CP is widely used as one of the most effective chemotherapeutic agents for treating ovarian, testicular and several other solid tumours, the biochemical mechanisms underlying the responsiveness of cancer cells to this drug remain unknown.

To date, most of the attention has been focused on the incidence and genetics of MMR defects while little is known about the expression levels of the MMR proteins and their significance in human cancer cell biology.

The aim of this study was to investigate the expression levels of hMSH2 and GTBP proteins by Western blotting in a series of previously untreated ovarian cancer patients. The correlation between hMSH2 and GTBP levels and clinico-pathological characteristics and response to CP-based chemotherapy have been also investigated.

**PATIENTS AND METHODS**

This study was conducted on 20 previously untreated primary ovarian cancer patients admitted to the Department of Gynecology at the Catholic University of Rome. Patient characteristics are listed in Table 1. The median age was 58 years (range 35–74). Fourteen patients had stage III and six had stage IV disease according to the International Federation of Gynecology and Obstetrics (FIGO) classification. One tumour was graded as well-differentiated (G1), three tumours as moderately differentiated (G2) and six as poorly differentiated (G3) (World Health Organization, 1979). Fifteen tumours were serous, three were endometrioid, one was mucinous and one was undifferentiated according to the World Health Organization histological typing of ovarian cancer (Serov and Scully, 1973). All patients underwent cytoreductive surgery. Surgical debulking was considered as optimal (residual tumour ≤ 2 cm) in 13 patients and non-optimal (residual tumour > 2 cm) in seven patients. Chemotherapy was instituted 2–3 weeks after surgery. All patients received chemotherapy containing CP (total CP dose = 500 mg).

Gynaecological examination, abdominopelvic ultrasonography, CA-125 assay and radiological investigations, if necessary, were performed monthly for the clinical assessment of response, which was recorded according to the World Health Organization criteria (World Health Organization, 1979). Approximately 1 month after the last course of chemotherapy, clinically complete responders underwent second-look laparoscopy. In laparoscopy negative cases, second-look laparotomy was performed for the assessment of pathological response. Of 17 patients evaluable for chemotherapy response, nine showed a pathologically complete response, two showed partial response and six showed no change of disease or disease progression. In three clinically negative patients, pathological assessment of response was not carried out because of patient refusal of a second surgery.

**Generation of anti-GTBP monoclonal antibodies**

The mouse anti-GTBP monoclonal antibodies (mAb) 66H6 and 21F10, both IgG1, were raised against the full-length recombinant GTBP protein. Female balb/c mice were immunized with four intraperitoneal and one intravrenal injections. The spleen cells were then fused with the myeloma line P3×63 Ag.8.653. Hybrids were selected with enzyme-linked immunosorbent assay (ELISA) and Western blots. They were then cloned by limiting dilution and the individual clones were again screened as above. The selected mAb-secreting lines were adapted to grow in roller bottles at a low percentage (1%) of fetal calf serum. The antibodies were purified from the culture medium by Gamma-plus Protein G Sepharose (Pierce).

**Table 1** Patient characteristics according to origin of the tumour cells and hMSH2 and GTBP levels

| Case | Origin of tumour cells | Age | Histotype | Stage | Grade | Residual tumour | Chemotherapy response | hMSH2 2 levels (a.u.) | GTBP levels (a.u.) |
|------|------------------------|-----|-----------|-------|-------|----------------|-----------------------|----------------------|--------------------|
| 1    | Ascites                | 49  | Serous    | III   | 3     | <2 cm         | CR                    | 3.009                | 1.623              |
| 2    | Solid tumour           | 68  | Serous    | IV    | 3     | <2 cm         | CR                    | 2.5                  | 1.3                |
| 3    | Ascites                | 60  | Serous    | IV    | 3     | <2 cm         | CR                    | 1.244                | 1.376              |
| 4    | Solid tumour           | 35  | Serous    | III   | 3     | <2 cm         | CR                    | 4.044                | 2.546              |
| 5    | Solid tumour           | 56  | Serous    | III   | 3     | >2 cm         | CR                    | 2.255                | 0.506              |
| 6    | Solid tumour           | 50  | Serous    | III   | 1     | ≤2 cm         | CR                    | 2.357                | 0.224              |
| 7    | Ascites                | 69  | Serous    | III   | 3     | ≤2 cm         | CR                    | 2.15                 | 1.88               |
| 8    | Ascites                | 72  | Serous    | III   | 3     | <2 cm         | CR                    | 1.406                | 1.765              |
| 9    | Ascites                | 51  | Serous    | IV    | 3     | <2 cm         | CR                    | 0.667                | 0.508              |
| 10   | Solid tumour           | 44  | Serous    | III   | 3     | <2 cm         | PR                    | 2.982                | 1.413              |
| 11   | Solid tumour           | 60  | Endometrioid | III  | 3     | <2 cm         | PR                    | 2.175                | 0.611              |
| 12   | Ascites                | 70  | Serous    | III   | 3     | ≤2 cm         | NC-P                  | 0                    | 0                  |
| 13   | Solid tumour           | 52  | Serous    | IV    | 3     | >2 cm         | NC-P                  | 0.389                | 0.28               |
| 14   | Ascites                | 74  | Undifferentiated | IV  | 3     | ≥2 cm         | NC-P                  | 0.852                | 0.961              |
| 15   | Solid tumour           | 46  | Serous    | IV    | 3     | <2 cm         | NC-P                  | 1.543                | 0.427              |
| 16   | Ascites                | 42  | Serous    | III   | 2     | <2 cm         | NC-P                  | 1.092                | 1.624              |
| 17   | Ascites                | 63  | Serous    | III   | 3     | >2 cm         | NC-P                  | 1.557                | 1.6                |
| 18   | Ascites                | 69  | Endometrioid | IV   | 3     | >2 cm         | n.d.                  | 1.63                 | 1.112              |
| 19   | Ascites                | 54  | Endometrioid | III  | 2     | >2 cm         | n.d.                  | 0.916                | 0.576              |
| 20   | Solid tumour           | 65  | Mucinous  | III   | 3     | >2 cm         | n.d.                  | 2.28                 | 1.174              |

CR: complete response; PR: partial response; NC-P: no change of disease or disease progression; N.D.: not determined.

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Preparation of primary tumour cells

Tumour specimens and ascitic fluid were aseptically obtained during surgery and tumour cells were prepared as previously described (Scambia et al, 1992) with minor modifications. Briefly, tumour cells were separated from ascitic fluid by centrifugation and from solid tumour biopsies by mechanical and biochemical (0.1% collagenase IV, Sigma, Milano, Italy) dissociation in Ham’s F-12 medium supplemented with antibiotics under aseptic conditions in a laminar flow hood. Cells were then filtered through a sterile gauze to remove cell clumps and passed trough 25-gauge needles in order to obtain a monocellular suspension. Cells were separated on a Ficoll-hypaque gradient. Collected cells were extensively washed in Hank’s balanced salt solution and resuspended in Ham’s F-12 medium. Tumour cells, assessed by immunohistochemical staining for cytokeratin on cytosin preparations, were detected in a percentage ranging from 64 to 92% (median: 73%).

Cell lines

The MMR-proficient human ovarian carcinoma cell line A2780, the human cervical cancer cell line HeLa, and the MMR-deficient human colorectal carcinoma cell lines LoVo (hMSH2-deficient) and HCT-15 (GTBP-deficient) (Boyer et al, 1995) were used in this study. All cultures were kindly provided by Dr P Karran (Imperial Cancer Research Fund, Herts, UK) and were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U ml–1 antibiotics and 0.3 m g ml–1 glutamine in a humidified 5% carbon dioxide incubator.

Preparation of cell lysates and Western blotting analysis

Total cellular proteins were isolated blind from primary tumour cells and from cell lines harvested during exponential growth, by lysing the cells in 1% sodium dodecyl sulphate (SDS), 20 mM EDTA, 10 mM Tris–HCl pH 8, 1.74 µg ml–1 phenylmethyl-sulphonyl fluoride, 2.5 µg ml–1 leupeptin, chymostatin, pepstatin and 0.2 µg ml–1 aprotinin. For hMSH2 and GTBP detection, 250 µg of each protein sample in 1 × SDS sample buffer were separated on a 6% SDS-polyacrylamide gel. After electroblotting, the polyvinylidene difluoride (Millipore Co., Bedford, MA) membranes were incubated with 6% non-fat dry milk in 1 × TBST (0.1 M Trizma base, 0.15 M sodium chloride, 0.05% Tween-20, pH 7.4) for blocking and then with the primary antibody in 3% non-fat dry milk in 1 × TBST. Following incubation with an alkaline phosphatase-conjugated goat anti-mouse antibody (Bio-Rad Laboratories, Hercules, CA, USA), visualization of the bound antibody was performed with the BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The samples were also analysed by Western blot for α-tubulin. Images of the blots were acquired with a Cohu CCD camera and quantification of the bands was performed by Phoretix 1 D (Phoretix International Ltd, Newcastle upon Tyne, UK). Band intensity was expressed as relative absorbance and hMSH2 and GTBP values were normalized to α-tubulin levels. Two to four different Western blot analyses were performed on our samples. The variability observed was never greater than 20%. The following mAbs were used: amino-terminal Ab-1 mouse anti-human MSH2 (Oncogene Science, Cambridge, MA, USA) (at 1:100 dilution), Ab-1 mouse anti-human α-tubulin (Oncogene Science, Cambridge, MA, USA) (at 1:100 dilution) and amino-terminal 21F10 and 10E10 mouse anti-human GTBP (generated as described above) (at 1:500 dilution).

Immunohistochemical analysis of hMSH2 in tissues and cell lines

Immunohistochemical analysis was performed using Ab-1 anti-hMSH2 antibody on frozen sections from histologically defined neoplastic ovarian tissues, normal human colonic mucosa and on cytospins of logarithmically growing A2780, LoVo and HCT-15 human cancer cell lines. Frozen tissues were obtained from surgical resections and stored at –80°C until used. Cryostat sections and cytospins were placed on SuperFrost slides and fixed in 4% paraformaldehyde for 10 min at room temperature. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in absolute methanol for 30 min. The primary antibody was applied for 1 h in 1:100 dilution and sequentially followed by biotinylated anti-mouse immunoglobulin and horseradish peroxidase-conjugated streptavidin (ABC-Vector Laboratories, Burlingame, CA, USA) and then incubated in the chromogenic substrate solution 3,3´-diaminobenzidine (Sigma, Milano, Italy) for 10 min. Sections were lightly counterstained with Harris’s haematoxylin, dehydrated, cleared and mounted. The percentage of positivity of the neoplastic cells was evaluated by counting at high magnification (1000 ×) at least five different areas of the samples. Sample analysis was performed blind.

Statistical analysis

Pearson’s correlation test was used to analyse the relationship between hMSH2 and GTBP levels. Kruskal–Wallis non-parametric test was used to analyse the distribution of hMSH2 and GTBP protein levels according to the clinico-pathological characteristics of the patients.

RESULTS

Characterization of anti-GTBP antibodies in cell lines

Among the anti-GTBP mAbs tested, 21F10 exhibited the best specificity and reactivity. Western blot analysis performed with 21F10 showed a main band of approximately 160 kDa corresponding to full-length GTBP in lysates from MMR-proficient A2780 and HeLa cell lines and in the hMSH2-defective LoVo cells (Figure 1). The amount of GTBP was considerably lower in LoVo lysate than in A2780 and HeLa lysates. Full-length GTBP was not detectable by Western blotting in the HCT-15 cells (Figure 1) which contain frameshift mutations in GTBP gene resulting in truncated proteins (Papadopoulos et al, 1995). However, this cell line showed a marked immunohistochemical nuclear reactivity with 21F10 anti-GTBP mAb (data not shown). This finding could indicate the preserved immunoreactivity of the truncated GTBP proteins since the frameshift mutations which generate a termination codon in HCT-15 cells (Papadopoulos et al, 1995) did not affect the amino-terminal region recognized by our mAb. In the absence of a characterized negative control for GTBP immunostaining in the literature, we considered the 21F10 anti-GTBP mAb as inadequate for precise immunohistochemical assessment of this protein.
Western blot analysis of hMSH2 and GTBP in ovarian cancer cells and relationship with clinico-pathological parameters

The expression levels of hMSH2 and GTBP in ovarian tumour cells obtained from ascites (n = 10) and solid tumours (n = 10) were examined by Western blot analysis with Ab-1 anti-hMSH2 and 21F10 anti-GTBP mAbs. A band of approximately 105 kDa, corresponding to hMSH2 and a band of 160 kDa, corresponding to GTBP, are observable in all samples but one (lane 8). In the lower panel, the samples were probed for α-tubulin.

Immunohistochemical analysis of hMSH2 in cancer cell lines and neoplastic ovarian tissues

Immunohistochemical analysis of hMSH2 with Ab-1 mAb was performed in the MMR-proficient A2780 ovarian cancer cells, in the hMSH2-defective LoVo colon carcinoma cells and in six ovarian cancer specimens obtained from primary surgery from a corresponding number of untreated ovarian cancer patients. Over 90% of A2780 cells exhibited an intense staining localized exclusively in the nuclei while LoVo cells, which carry a deletion in both alleles of the hMSH2 gene (Umar et al, 1994), did not show immunoreactivity (Figure 4 A and B respectively). In all samples of primary ovarian cancer, a specific nuclear immunoreaction with Ab-1 was observed in the vast majority of the neoplastic cells (over 80% of positive cells) (Figure 4 C, D). In three specimens of normal human colonic mucosa used as a positive control for Ab-1 staining, immunohistochemistry revealed that hMSH2 immunoreactivity was confined to the glandular epithelium (data not shown) as previously described by several authors using different anti-hMSH2 antibodies (Wilson et al, 1995; Leach et al, 1996).

DISCUSSION

This is the first study analysing the expression levels of hMSH2 and GTBP proteins in a series of primary ovarian cancers. We...
demonstrated that hMSH2 protein is exclusively localized in the nuclear compartment and is expressed in the vast majority of neoplastic cells from primary ovarian tumours. These results are consistent with those of Brown et al (1997), and with previous demonstrations that microsatellite instability occurs with a low prevalence in advanced stage ovarian cancer (King et al, 1995; Arzimanoglou et al, 1996), indicating that the majority of tumour cells from these patients carry a functional hMSH2 genes. Western blotting analysis revealed that hMSH2 and GTBP display a wide range of expression levels, suggesting that both these proteins could play a role in the biology of ovarian tumour cells.

It has been previously demonstrated by Palombo et al (1995) that the amount of hMSH2 and GTBP in the GTBP-deficient HCT-15 and in the hMSH2-deficient LoVo cells, respectively, was considerably lower that that in the MMR-proficient HeLa cells, thus leading these authors to hypothesize that the two proteins are unstable when not in a complex. Even though we cannot rule out this possibility, the direct correlation between hMSH2 and GTBP expression levels found in primary ovarian cancer cells is likely to suggest that the expression of these two proteins may be regulated by a common mechanism.

Only hMSH2 levels were found to significantly differ according
to the origin of the tumour cells, suggesting that hMSH2 levels could be related to different biological characteristics of ascites-derived with respect to solid tumour-derived cells. Ovarian tumour cells which exfoliate from the surface of the solid tumour are likely to develop distinct biological characteristics since they lose cell–cell and cell–substrate interactions and, on the other hand, develop the ability to grow in the intraperitoneal microenvironment, which is particularly rich in cytokines and growth factors (Kutteh and Kutteh, 1992), and to form metastatic implants on peritoneal surface. However, in order to investigate whether primary tumour and ascites represent two biologically distinct entities, a multivariate analysis including other biologically relevant parameters is needed and thus a larger series of cases is required to adequately address this issue.

No statistically significant differences were found in the distribution of hMSH2 and GTBP levels according to age of the patients, grade of differentiation, histotype and surgical debulking. Conversely, we found that low hMSH2 protein levels were associated with poor chance of response to CP-based regimens. Our findings cannot be seen merely as a consequence of the reduced hMSH2 levels in stage IV patients since no difference in the type of response according to stage of disease was found in our series. Two different hypotheses could be considered: first, it has been demonstrated that increased hMSH2 protein expression is associated with the entrance of resting cells into the cell cycle (Marra et al., 1996). It is therefore conceivable that cancers expressing low hMSH2 levels have a low proliferative fraction, which is commonly considered to imply a low susceptibility to chemotherapy. Alternatively, it has been demonstrated that the amount of hMSH2 protein directly correlated with mismatch G-T binding activity and that both parameters inversely correlated with the level of drug resistance in various Chinese hamster ovary cells showing different degrees of resistance to methylating agents (Dosch et al., 1998). In this context, Mello et al. (1996) previously hypothesized that, similarly to the biochemical pathway proposed for methylating agents, a mechanism that involves a futile cycle of excision and resynthesis of damaged DNA and ultimately results in cell death, could also be operative in the processing of CP adducts. According to this model, the binding of hMSH2 to CP-modified DNA would trigger the recruitment of other MMR protein(s) causing misdirected repair attempts at sites of CP damage. This abortive repair activity and/or the subsequent accumulation of DNA strand breaks could provide a signal resulting in growth arrest in the G2 phase of the cell cycle and subsequently cell death. Thus, cancer cells with a relatively low content of hMSH2 would be unable to increase DNA damage to an extent sufficient to trigger cell death.

In conclusion, our study suggests a possible involvement of hMSH2 in ovarian cancer cell biology and in influencing the susceptibility to chemotherapeutic agents whereas the role of GTBP remains unclear. However, further studies are required to allow a better characterization of the function of the MMR system in human cancer.

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