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Original Paper

Bcl-2 as a Predictor of Chemosensitivity and Prognosis in Primary Epithelial Ovarian Cancer

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This retrospective study of ovarian cancer aimed to elucidate whether expression of apoptosis-related proteins, bcl-2, p53 or MDM-2, is associated with resistance to chemotherapy, especially cisplatin (CDDP) based chemotherapy. Expression of bcl-2, p53 and MDM-2 was assessed by immunohistochemical staining of tumour tissues collected at initial surgery prior to treatment with CDDP-based chemotherapy. Among 66 patients with advanced ovarian cancer with measurable tumour following surgery and evaluable for response to chemotherapy, 42, 45 and 56% were positive for bcl-2, p53 and MDM-2, respectively. Significantly fewer tumours of patients who had a complete response to chemotherapy (CR) showed positivity for bcl-2 (2/20) than for p53 (6/20) and MDM-2 (8/20, $P<0.001$). There was an inverse correlation between bcl-2 staining and initial response to chemotherapy, especially in serous and endometrial adenocarcinomas. In patients with stage III–IV, serous or endometrioid adenocarcinomas, significantly poorer survival was seen for those with bcl-2 positive tumours than those with negative bcl-2 staining ($P=0.0064$). p53 and MDM-2 were not correlated with initial response to chemotherapy. Multivariate analysis revealed that bcl-2, residual tumour size and histology were significant independent prognostic factors. These results suggest that bcl-2 can be a possible predictor of response to chemotherapy and prognosis in patients with advanced ovarian carcinoma.

Key words: bcl-2, cisplatin, ovarian cancer, p53, MDM-2, cisplatin resistance, apoptosis
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INTRODUCTION

THE EFFICACY of cancer chemotherapy is restricted by the ability of tumours to resist or develop resistance to treatment. Ovarian cancer shows high response rates to first-line chemotherapy but is characterised by recurrence and the development of resistance to chemotherapy, so that prognosis is poor, with only a limited number of patients (approximately 25%) surviving 5 years. Resistance to chemotherapy has been associated with decreased susceptibility to apoptosis [1], raising the possibility that cell death determinants may influence the outcome of treatment.

It has been shown that the bcl-2 protein promotes cell survival [2] by inhibiting the process of programmed cell death or apoptosis [3, 4]. A variety of carcinomas, including ovarian carcinoma, resistant to anticancer drugs express bcl-2, suggesting that bcl-2 may protect cancer cells from programmed cell death induced by a variety of antitumour agents including cisplatin [5, 6]. Therefore, expression of bcl-2 by tumours may confer resistance to chemotherapy by enabling cells to avoid apoptosis. So far it is not known whether bcl-2 and its role in the regulation of apoptotic cell death are of importance in ovarian carcinomas.

It is accepted that overexpression of *TP53* as detected by immunohistochemistry is usually due to an underlying mutation of the *TP53* gene, leading to the expression of an abnormal and stabilised protein [7]. However, loss of p53 function, together with stabilisation of the protein, may be caused by other mechanisms, such as interaction of the wild-type p53 with other cellular proteins such as MDM-2. Thus, although immunohistochemical detection of p53 does not necessarily indicate expression of a mutated protein, it may be a useful marker for the presence of a functionally abnormal *TP53* [8].

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The *mdm-2* gene is overexpressed in several human tumours. In response to a number of DNA-damaging agents, p53 has been shown to transactivate concurrently genes responsible for cell cycle arrest and the mdm-2 gene, which encodes proteins that are capable of binding to and inactivating p53 [9, 10]. MDM-2 can inhibit p53 activity relating to cell cycle arrest, DNA repair and apoptosis. Post-translational modification of MDM-2 can influence the interaction between MDM-2 and p53.

Given the apparent role of bcl-2, p53 and MDM-2 expression in oncogenesis and resistance to chemotherapy, we decided to investigate whether bcl-2, p53 and MDM-2 are expressed in ovarian carcinoma and whether expression of these genes has any prognostic significance or relationship to a patient's response to chemotherapy.

PATIENTS AND METHODS

Tissues and patients' characteristics

Formalin-fixed, paraffin-embedded tissue samples of primary ovarian carcinomas were collected from the National Defense Medical College Hospital, Japan. Archival materials from 105 patients with primary ovarian carcinoma were studied and their characteristics are shown in Table 1. All patients received primary surgery and at least six cycles of chemotherapy consisting of cyclophosphamide, doxorubicin and cisplatin, (so-called 'CAP'). Of these 105 patients, only 66 with measurable tumours were evaluable for response to chemotherapy (see Table 1).

Response definition

Clinical complete response (CR) was defined as the complete disappearance of all clinically measurable disease. Clinical partial response (PR) was defined as a $\geq$50% decrease in the sum of the products of cross-sectional diameters of all measurable lesions that lasted at least 1 month. Stable disease (SD) was defined as any condition other than an objective response or progressive disease. Progressive disease (PD) was defined as a $\geq$25% increase in the sum of the products of the cross-sectional diameters of all measurable lesions. WHO criteria was used to assess clinical response.

Table 1. *Patients' characteristics*

Age	Median 51 (range 18–78 years)
Histological type	
Serous	52
Endometrioid	18
Clear	25
Mucinous	8
Undifferentiated	2
FIGO stage	
I	16
II	16
III	55
IV	18
Response to chemotherapy	
CR	20
PR	18
SD	10
PD	18

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Responder had CR or PR and non-responder had SD or PD.

Immunohistochemistry

Tissue sections (4 μm) from each case were mounted on Super/Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.). Tissue sections were cut 1–3 days prior to immunostaining. Sections were deparaffinised in three changes of xylene and hydrated in one change each of absolute ethanol, 95% ethanol and 70% ethanol for 2 min in each solution. Specimens were then microwaved in 1 litre of a sodium citrate buffer (pH 6) for 45 min for heat-mediated antigen retrieval. A 1% solution of hydrogen peroxide in methanol was used to block endogenous peroxidase activity before transferring the sections into phosphate-buffered saline (PBS) (0.1 M; pH 7.2). Non-specific binding was blocked by incubation with horse non-immune serum for 20 min. The slides were incubated overnight at 4°C with primary antibody (anti-bcl-2, anti-p53 or anti-MDM-2) at an appropriate dilution (for anti-bcl-2, 1:50; for anti-p53, 1:50; and for anti-MDM-2, 1:100). The primary antibodies used in this study

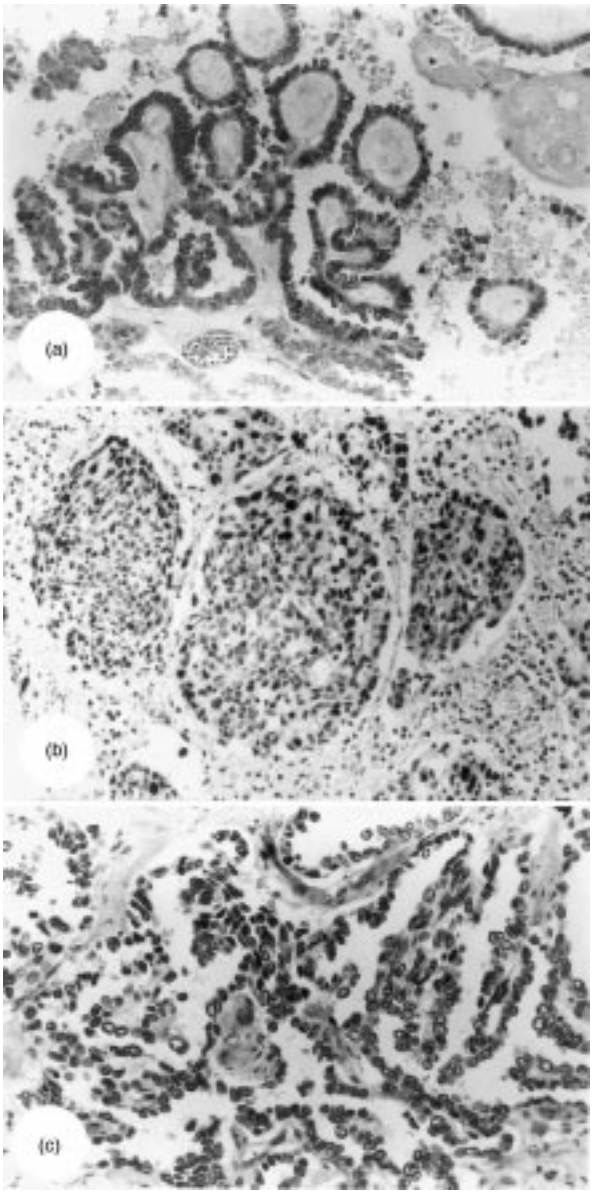

Figure 1. Examples of immunohistochemical staining patterns. Note the nuclear staining in the positive cells. (a) bcl-2, (b) p53, (c) MDM-2.

were a mouse monoclonal anti-bcl-2 antibody (clone 124, Dako, Glostrup, Denmark), a mouse monoclonal anti-p53 antibody (clone DO-7, Dako, Glostrup, Denmark) and a mouse monoclonal antibody NCL-MDM-2 (Nova Castra Labs, Newcastle, U.K.). The slides were then incubated with a biotinylated rabbit anti-mouse immunoglobulin for 30 min at room temperature. They were subsequently incubated with avidin-biotin peroxidase complex (Vectastain Elite kit, Vector Laboratories, Burlingame, California, U.S.A.). The sections were counterstained with haematoxylin. Omission of the primary antibody was performed as a negative control. All slides were evaluated for immunostaining without any knowledge of the clinical outcome or other clinicopathological data. Nuclear staining for bcl-2, p53 and MDM-2 were evaluated (Figure 1). When 5% or more for bcl-2 (for nuclear, but not cytoplasmic staining) and 10% or more for p53 and MDM-2 of the tumour cells showed immunoreactivity, the result was interpreted as positive. We examined nuclear rather than cytoplasmic staining for bcl-2 because it has been reported that bcl-2 binds to chromatin in the nucleus and inhibits apoptosis [21, 22]. Therefore, nuclear localisation of bcl-2 might be related to inhibition of apoptosis, subsequently resulting in resistance to chemotherapy. Sakuragi and colleagues [23] reported that nuclear bcl-2 expression in endometrial carcinoma was associated with shorter survival than that of patients with cytoplasmic bcl-2 expression. Therefore, in the present study, we defined positivity as $\geq$5% nuclear bcl-2 staining but not the cytoplasmic staining. Lymph nodes for bcl-2, small cell lung cancer for p53 and liposarcoma for MDM-2 were used as positive controls.

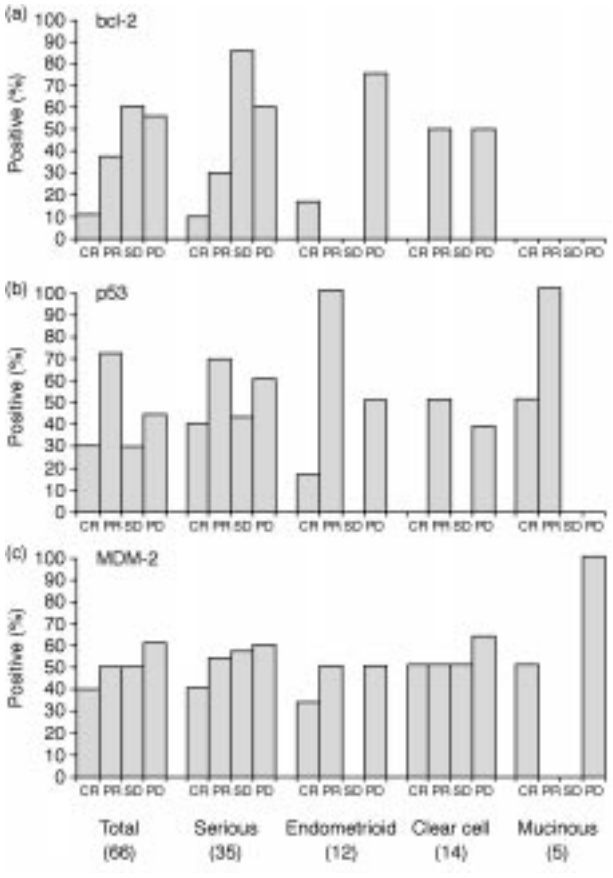

Figure 2. Relationship between histology, response to chemotherapy and expression of (a) bcl-2, (b) p53 or (c) MDM-2 in 66 advanced ovarian cancer patients with measurable tumour. CR, complete response; PD, partial response; SD, stable disease; PD, progressive disease.

Statistical analysis

The various parameters were compared by contingency-table analysis or chi-square tests. For survival analyses Kaplan–Meier curves were calculated and log-rank tests were used. Multivariate analysis was performed according to Cox's proportional-hazard model. P values <0.05 were considered statistically significant.

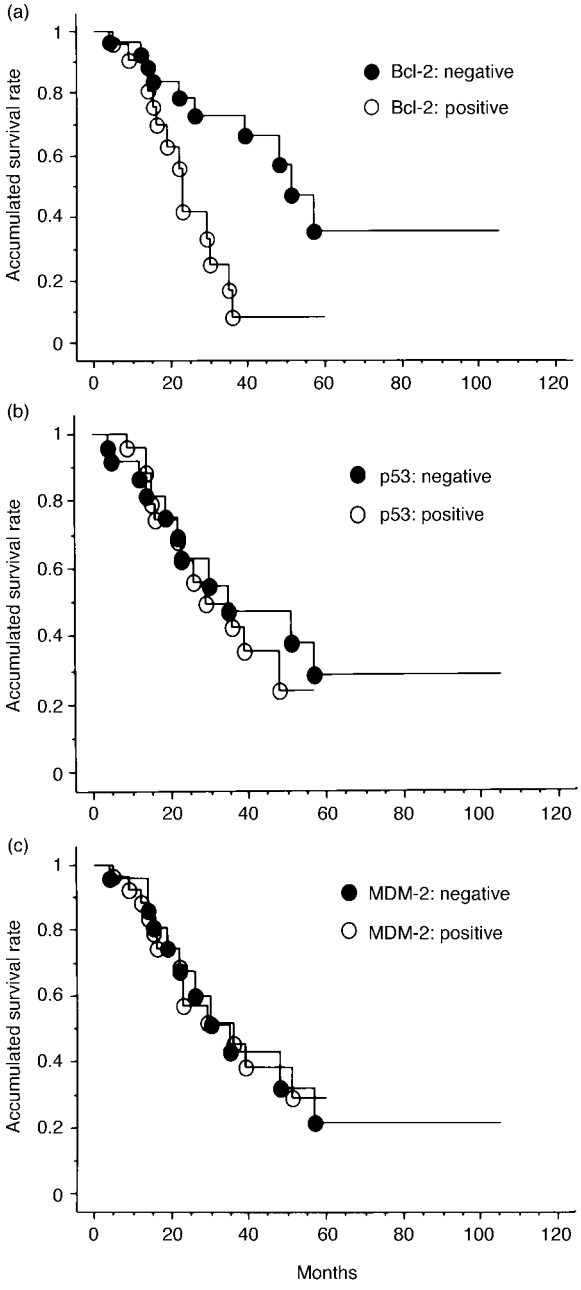

Figure 3. Survival rate of 51 patients with stage III, IV serous and endometrioid adenocarcinoma according to expression of bcl-2, p53 or MDM-2. Patients with negative bcl-2 tumour were significantly ($P=0.0064$) longer survived than those with positive bcl-2 tumour (a). There was no significant difference between survival rates of patients with negative and positive p53 tumour (b) or negative and positive MDM-2 tumour (c).

Table 2. Positivity of ovarian carcinomas for bcl-2, p53 and MDM-2

	bcl-2 positive *n* (%)	p53 positive *n* (%)	MDM-2 positive *n* (%)
All histological type	42 (41)	46 (70)	54 (82)
Serous ($n=52$)	26 (50)	29 (56)	29 (56)
Endometrioid ($n=18$)	7 (39)	8 (44)	8 (44)
Clear ($n=25$)	7 (28)	6 (24)	13 (52)
Mucinous ($n=8$)	1 (13)	2 (25)	2 (25)
Undifferentiated ($n=2$)	1 (50)	1 (50)	2 (100)
FIGO stage			
I ($n=16$)	4 (25)	6 (38)	7 (44)
II ($n=16$)	7 (44)	7 (44)	6 (38)
III ($n=55$)	21 (38)	21 (38)	25 (45)
IV ($n=18$)	10 (56)	12 (67)	16 (89)
Residual tumour			
<2 cm ($n=64$)	23 (36)	20 (31)	28 (44)
≥ 2 cm ($n=41$)	19 (46)	26 (63)	26 (63)
Response to chemotherapy ($n=66$)	23 (35)	30 (45)	33 (50)
CR ($n=20$)	2 (10)*	6 (30)	8 (40)
PR ($n=18$)	7 (39)	13 (72)	9 (50)
SD ($n=10$)	6 (60)	3 (30)	5 (50)
PD ($n=18$)	10 (56)	8 (44)	11 (61)

For abbreviations see Table 1. *$P<0.001$, compared with p53 and MDM-2.

RESULTS

Table 2 shows the positivity of ovarian carcinomas for bcl-2, p53 and MDM-2 by histological type, FIGO stage and residual tumour size after initial surgery. Positivity of these protein markers were higher (non-significant) in patients with residual tumour ≥ 2 cm after initial surgery. The relationship between response to chemotherapy and expression of bcl-2, p53 or MDM-2 in 66 patients with measurable tumour after initial surgery is shown in Table 2. Significantly fewer patients with a complete response (CR) to chemotherapy showed positivity for bcl-2 than for p53 and MDM-2 ($P<0.001$). Positivity for bcl-2 was inversely correlated with response to chemotherapy, whilst that of p53 and MDM-2 had no correlation (Figure 2). In particular, positivity for bcl-2 was increased with poorer response to chemotherapy in serous and endometrioid adenocarcinoma, but not clear cell or mucinous adenocarcinoma (Figure 2). Survival of patients with stage III–IV serous or endometrioid adenocarcinoma negative for bcl-2 was significantly better ($P=0.0064$) than that for those positive for bcl-2 (Figure 3). Although none of these particular mucinous adenocarcinoma were positive for bcl-2, expression of bcl-2, p53 and MDM-2 seemed to have no correlation with histological types either in the subset of 66 (data not shown) nor in the total sample of 105 (Table 2). At least 63% of patients whose tumours were negative for bcl-2 responded to chemotherapy, irrespective of p53 or MDM-2 positivity (Table 3). Those negative for bcl-2 and MDM-2 had a CR rate of 50%. Patients with tumours positive for bcl-2 and negative for p53 or MDM-2 showed markedly poor response rates (Table 3). Multivariate analyses by Cox's regression model revealed that residual tumour size (≥ 2 versus <2 cm), histology (clear cell or mucinous versus serous or endometrioid adenocarcinoma) and bcl-2 (positive versus negative) were independent prognostic factors for predicting overall survival (Table 4). As shown in Table 5, patients with stage III–IV serous or endometrioid adenocarcinomas positive for bcl-2 and with ≥ 2 cm residual tumour showed no complete response to chemotherapy, whilst all those with

Table 3. Correlation between apoptosis-related protein expression pattern and response to chemotherapy

Protein expression		CR (%)	PR (%)	CR + PR (%)	SD (%)	PD (%)
bcl-2 (−)	p53 (−) ($n=26$)	12 (46)	5 (19)	17 (65)	3 (12)	6 (23)
	p53 (+) ($n=17$)	5 (29)	8 (47)	13 (76)	1 (6)	3 (18)
	MDM-2 (−) ($n=24$)	12 (50)	6 (25)	18 (75)	3 (13)	3 (13)
	MDM-2 (+) ($n=19$)	5 (26)	7 (37)	12 (63)	1 (5)	6 (32)
bcl-2 (+)	p53 (−) ($n=10$)	1 (10)	0 (0)	1 (10)	4 (40)	5 (50)
	p53 (+) ($n=13$)	1 (7.7)	4 (31)	5 (38)	3 (23)	5 (39)
	MDM-2 (−) ($n=9$)	0 (0.0)	2 (22)	2 (22)	3 (33)	4 (44)
	MDM-2 (+) ($n=14$)	2 (14.3)	2 (14)	4 (29)	4 (29)	6 (43)

For abbreviations see Table 1. Since only bcl-2 was associated with survival results as shown in Figure 2, bcl-2 status was examined in comparison with p53 status and MDM-2 status.

Table 4. Multivariate analysis of prognostic factors for predicting overall survival by Cox's proportional hazards model

Covariate	Hazard ratio	95% CI	*P* value
Age (≥ 51 versus <51) years	0.540	0.260–1.119	0.0973
Residual tumour size (≥ 2 cm versus <2 cm)	6.885	2.622–18.082	0.0001
Histology (C + M versus S + E)	10.085	4.151–24.499	0.0001
bcl-2 (positive versus negative)	3.097	1.463–6.556	0.0031
p53 (positive versus negative)	0.717	0.330–1.560	0.4015
MDM-2 (positive versus negative)	1.392	0.649–2.985	0.3953

Results of 66 advanced ovarian cancer patients with measurable tumour. CI, confidence interval; S, serous adenocarcinoma; E, endometrioid adenocarcinoma; C, clear cell adenocarcinoma; M, mucinous adenocarcinoma.

Table 5. *Relationship between bcl-2 expression and/or residual tumour size and response to chemotherapy in stage III, IV patients with serous and endometrioid adenocarcinoma*

Factors	CR (%) $n=16$	PR (%) $n=15$	SD (%) $n=7$	PD (%) $n=9$
bcl-2 positive				
RT≥2 cm	0 (0)	4 (27)	5 (71)	2 (22)
RT<2 cm	2 (13)	0 (0)	1 (14)	4 (44)
bcl-2 negative				
RT≥2 cm	3 (19)	9 (60)	1 (14)	3 (33)
RT<2 cm	11 (69)	2 (13)	0 (0)	0 (0)

For abbreviations see Table 1. RT, residual tumour.

tumours negative for bcl-2 and with <2 cm residual tumour responded completely or partially to chemotherapy.

DISCUSSION

The products of the *bcl-2* and *TP53* genes are involved in the regulation of apoptosis and proliferation and have been associated with prognosis in several malignancies, including primary ovarian carcinoma [11–15]. MDM-2 can inhibit p53 activity relating to cell cycle arrest, DNA repair and apoptosis [16]. MDM-2 protein may act as a negative regulator of cisplatin-induced apoptosis and, therefore, confer cisplatin-resistance [17]. The susceptibility of tumour cells to apoptotic cell death following chemotherapy is a major determinant in the outcome of therapy.

In this retrospective study, the expression of bcl-2 and p53 was low in mucinous and clear cell adenocarcinoma, which have been reported to have a poor prognosis [18] compared with the other histological types (Table 2), although the reason for this is still unknown. Positivity for bcl-2, p53 or MDM-2 was higher in patients with stage IV tumours or with large residual tumours (Table 2). It is possible that the acquisition of mutations in tumour-suppressor genes such as *TP53* and the anti-apoptotic effects of bcl-2 and MDM-2 promote progression of the tumour and select malignant phenotypes with cisplatin resistance. In patients with advanced stage III and IV tumours, positivity for bcl-2, p53 and MDM-2 was 42, 45 and 56%, respectively, similar to previously reported results [12, 19]. Bcl-2, but not p53 and MDM-2 expression was correlated with response to chemotherapy (Figure 2). Many papers have reported that positivity of bcl-2 is positively correlated with prognosis of ovarian cancer [12, 15, 20], but few papers had described this relationship between positivity of bcl-2 and response to chemotherapy. To the best of our knowledge, the present study is the first report to show that nuclear bcl-2 expression in ovarian serous and endometrioid adenocarcinoma is associated with poor prognosis. In addition, multivariate analyses of prognostic factors for predicting overall survival revealed that residual tumour size, histology and bcl-2 were significant independent prognostic factors in patients treated with combination chemotherapy including cisplatin. The finding on residual tumour suggests that when tumours are removed at primary surgery and are shown to be positive for bcl-2 nuclear staining, it may be more appropriate to select paclitaxel for chemotherapy rather than a cisplatin-containing regime, although further studies are needed to confirm this result.

In conclusion, we have reported that nuclear (but not cytoplasmic) staining of bcl-2 in primary epithelial ovarian carcinoma, especially serous and endometrioid adenocarcinoma, is associated with poor response to cisplatin-based chemotherapy (not including paclitaxel). These results suggest that identification of such new prognostic markers would be of great importance in identifying individuals who may benefit from new and more effective therapy.

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