Modern Pathology (2008) 21, 1002–1010

www.modernpathology.org

Immunohistochemical analysis of possible chemoresistance markers identified by micro-arrays on serous ovarian carcinomas

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Using the DNA microarray technology, we have identified genes that are differentially expressed in chemosensitive and chemoresistant ovarian serous papillary carcinomas and could potentially distinguish ovarian cancer patients based on their response to chemotherapy. The present study aims to evaluate the clinical usefulness of overexpression of selected genes by immunohistochemistry. Our cohort included 158 women who were operated on and received chemotherapy for an advanced serous papillary ovarian carcinoma (FIGO stages III and IV). The end point used in this study was progression-free survival. Immunohistochemistry was performed on microarray blocks containing all 158 cases. Twelve commercially available antibodies were selected. Of them, 10 corresponded to differentially expressed genes in our micro-array study and p53 and Ki67 were included. Antibodies were obtained for the following selected genes: *GSTA1*, *MMP1*, *FOSB*, *CTSL2*, *HSP10*, *CD36*, *CXCL2*, *RBBP7*, *Siva*, and *PTGDS*. Cox proportional hazards models, adjusted for standard risk factors, were used to estimate the associations between the markers and progression-free survival. No association was found between mRNA level and protein expression by immunohistochemistry. In multivariate analyses, patients whose tumors overexpressed HSP10 had a lower risk of progression than those with low expression (HR: 0.6; CI: 0.42–0.87; $P=0.007$). High level of proliferation (Ki67) tended to be associated with a lower risk of progression (HR: 0.72; CI: 0.51–1.03; $P=0.07$) whereas MMP1 overexpression tended to be associated with a higher risk of progression (HR: 1.61; CI: 0.94–2.79; $P=0.08$). Our study shows that gene expression analysis coupled with immunohistochemistry allowed the identification of HSP10 as an independent factor of progression-free survival.
Modern Pathology (2008) **21,** 1002–1010; doi:10.1038/modpathol.2008.80; published online 23 May 2008

Keywords: serous ovarian carcinoma; chemoresistance; HSP10; DNA microarray; immunohistochemistry

Ovarian cancer is responsible for more cancer deaths among women in the Western world than any other gynecologic malignancy.[1] An initial surgical approach is essential for aggressive cytoreduction and proper staging of the disease, since minimal residual tumor after surgery is a major factor of better response to chemotherapy and survival.[2] Intravenous combinated chemotherapy with taxol plus carboplatin is the current regimen of choice for the treatment of advanced ovarian cancer and is followed by a 50% complete pathologic remission rate.[3]

Resistance to chemotherapy is, however, a major concern. Indeed, although significant proportions of women respond to chemotherapy, the majority of responders (approximately 60–75%) eventually relapses and dies from recurrent disease while 20–30% of patients never experience a clinical remission.[4] Chemotherapy resistance in ovarian

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Received 14 January 2008; revised 21 March 2008; accepted 24 March 2008; published online 23 May 2008

cancer is broad and encompasses diverse, unrelated drugs, suggesting more than one mechanism of resistance. Until now, very few markers were found to predict tumor response to chemotherapy and prognosis in ovarian cancer.[5–7] Recent advances in microarray technology led to identification of gene signatures that can help to improve diagnosis of ovarian cancer[8] and *in vitro* drug resistance[9,10] but not clinical response to chemotherapy.[11]

We recently analyzed the gene expression patterns in advanced (FIGO stages III and IV) primary serous papillary carcinomas of the ovary displaying different response to first line chemotherapy in an attempt to identify specific molecular signatures associated with clinical response to chemotherapy.[12,13] Initially, the expression profiles of 15 chemoresistant serous papillary carcinomas (recurrence $\leq$6 months) and 10 chemosensitive serous papillary carcinomas (recurrence $\geq$30 months) tumors were independently analyzed which allowed the identification of 155 genes with different expression in the chemoresistant or the chemosensitive phenotype. The 155 genes differently expressed at a *P*-value cutoff of 0.01 were upregulated or downregulated at least 2-fold in chemoresistant tumors in comparison with chemosensitive tumors. Functional classes of these differently expressed genes mainly include metabolism (30%), cell growth and maintenance (18%), signal transduction (12%), immune response (12%), cell organization and biogenesis (11%), transport (9%) and apoptosis (3%); the remainder (5%) have unknown functions.

This experiment prompted us to test the hypothesis that the detection of corresponding markers at the protein level by immunohistochemistry may prove clinically applicable to the daily practice of pathology to predict the response to chemotherapy. We decided to analyze 10 markers for which commercial antibodies were available on an independent, uniform cohort of patients with serous papillary carcinomas.

Materials and methods

Patients included in this study were operated between January 1998 to December 2003 for an advanced ovarian cancer at the CHUQ-L'Hôtel-Dieu hospital in Quebec City, Canada. Inclusion criteria were: serous papillary carcinoma histology, FIGO Stages III or IV and chemotherapy received after the surgery. The grade was evaluated using criteria defined by Silverberg,[14] which was in use in our institution during the accrual period. Clinical response to chemotherapy was evaluated using modified RECIST criteria.[15] The follow-up was available until death or to the date the study was closed (31 July 2004). The study was approved by the Institutional Ethical Committee.

One representative block of each ovarian tumor was selected for the preparation of the tissue arrays. Three 0.6 mm cores of tumor were taken from each tumor block and placed, 0.4 mm apart, on a recipient paraffin block using a commercial tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). The cores were randomly placed on one of three recipient blocks to avoid immunohistochemistry evaluation biases. Four micron-thick sections were cut for the hematoxylin–eosin (H&E) staining and immunohistochemistry analyses.

The antibodies were selected based on the capacity of the corresponding gene to predict ovarian cancer prognosis in our previous microarray study.[13] A serious constraint was their commercial availability. The antibodies are presented in the Table 1. P53 and Ki-67 were included in our study because of their general interest in oncology. Immunohistochemistry staining was performed using the avidin-biotin complex method. Briefly, one representative 4 μm tissue section was cut from the tissue array blocks. Sections were deparaffinized and rehydrated in graded alcohols, then incubated with blocking serum for 20 min. The antibody dilutions, retrieval method, and incubation conditions are detailed in Table 2. Sections were incubated with a biotinylated secondary antibody (Dako, Carpinteria, CA, USA) and then exposed to a streptavidin complex (Dako, Carpinteria, CA, USA). Complete reaction was revealed by 3–3′ diaminobenzidine and the slide was counterstained with hematoxylin. Positive controls used in each case are described in Table 2. Negative controls consisted of tissue sections incubated with phosphate-buffered saline (0.16 M, pH $\sim$7.5) instead of the primary antibody. For three antibodies (CD36, FOSB and MMP1) the staining was weak and we used the catalyzed signal amplification system (Dako, Carpinteria, CA, USA).

Positive staining was defined when more than 10% of cells expressed the marker, except for Ki-67 for which 20% was defined as a threshold.[16] The relationship between marker expression and patients' age, tumor grade, tumor size and the type of chemotherapy received was evaluated by the χ^2 *t*-test. Cox regression analyses were performed to estimate the association between tumor expression and progression free survival. Progression free survival was defined as the time from surgery to the first observation of disease progression, recurrence or death. Multivariate analyses, taking into account standard or strongly associated prognostic variables, were performed to identify independent prognostic factors. A significant association was considered when *P*-value was below 0.05 and a trend for values between 0.05 and 0.1. Kaplan–Meier curves were done to show progression-free survival for each marker. The immunohistochemistry staining was analyzed independently by two pathologists (BT, IP) blinded to clinical data and progression.

Table 1 Antibodies used in the study

No.	Gene name/genbank	Antibody	Clone	Description
1	*PTGDS/AK075333*	Prostaglandin D synthase	Cayman[a] 10004344	Mouse polyclonal antibody against prostaglandin D synthase (lipocalin-type, β trace) protein
2	*CD36/M98398*	CD36	SCBT[b] sc-21772	Mouse monoclonal antibody against human leukocytes CD36 antigen
3	*CXCL2/BC015753*	MIP2	SCBT sc-1388	Goat polyclonal antibody against an epitope mapping at the C terminus of MIP-2
4	*FOSB/NM_006732*	FOSB	SCBT sc-8013	Mouse monoclonal antibody against amino acids 75–150 mapping at the N terminus of Fos B of human origin
5	*HSPE1/BC023518*	HSP10	SCBT sc-20958	Rabbit polyclonal antibody against an epitope corresponding to amino acids 1-102 representing full length HSP10 of human origin
6	*CTSL2/AB001928*	Pan-cathepsin	SCBT sc-25537	Rabbit polyclonal antibody against an epitope corresponding to amino acids34–333 mapping at the C terminus of cathepsin L of human origin
7	*MMP1/NM_002421*	MMP1	SCBT sc-21731	Mouse monoclonal antibody against amino acids 366–376 of MMP-1 of human origin
8	*GSTA1/S49975*	GST	Abcam[c], ab856-6	Mouse monoclonal antibody against GST3 dimeric protein consisting of two identical 27 kDa subunits
9	*Siva/U82938*	Siva	SCBT sc-7436	Goat polyclonal antibody against an epitope mapping near the N terminus of Siva of human origin
10	*RBBP7/NM_002893*	RbAp46	Abcam, ab3535	Mouse monoclonal antibody against retinoblastoma-associated protein 46 (RbAp 46)
11		Ki67	Dako[d], MIB1	Mouse monoclonal antibody against the Ki-67 antigen and recombinant fragments of the Ki-67 molecule
12		p53	Dako, PAb240	Mouse monoclonal antibody against an epitope of the p53 protein only exposed on mutant forms in which structural mutations have altered the protein conformation

[a]Cayman Chemicals, Ann Arbor, MI, USA.
[b]Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.
[c]Abcam Inc., Cambridge, MA, USA.
[d]DakoCytomation Inc., Carpinteria, CA, USA.

Results

The Study Population

During the accrual period, we retrieved 235 consecutive cases operated in our hospital for an advanced serous papillary ovarian carcinoma, stage III and IV. Seventy-seven cases were excluded. Of them six refused chemotherapy, 10 died before the beginning of chemotherapy, clinical information were incomplete in 34 cases, in one case the stage was uncertain, eight patients had preoperative chemotherapy, one had a tumor of uncertain origin and 17 were still under chemotherapy at the last follow-up. A total of 158 cases responded to all inclusion criteria.

Table 3 shows the major clinical characteristics of the patients. The age ranged from 28 to 88 years (median: 61 years). Tumors were mainly grade 3 (67%) and stage III (78%). Seventeen patients had a second cancer, of them 10 were from the breast, three from the colon, one from the endometrium, two from the skin and there was one malignant lymphoma. A majority of patients (71%) received an intravenous combination of platinum and taxol, which was associated with a lower risk of progression compared to other combinations (Hazard ratio: 0.44 [0.26; 0.74]; $P=0.002$). The median baseline CA125 was 800 U/ml and a higher than average CA125 level was associated with increased risk of progression (Hazard ratio: 1.72 [0.99; 2.97]; $P=0.05$).

Figure 1 shows the status of the patients at the end of the chemotherapy regimen and their evolution during the study. Ninety-seven patients had a complete response, of whom 77 underwent recurrences and 32 finally died of their disease. Twenty-nine patients had partial response or stable disease at the end of the chemotherapy, of whom 21 had progression and 12 died. Thirty-two patients had progression of their cancer under chemotherapy and 21 died. The median follow-up period of the cohort was 26.1 months. Fifty percent of the patients had a progression or a recurrence within the first 12 months of follow-up. At 5 years, only 13% of patients are recurrence-free and 43% are alive.

Figure 2 shows examples of immunostaining with the different antibodies used in this study. MMP1 gave a cytoplasmic granular staining in cancer cells while stromal cells were negative (Figure 2a); CD36 was expressed in the cytoplasm of cancer cells and stromal cells were negative (Figure 2b); HSP10 showed a cytoplasmic and granular staining of both cancer and stromal cells (Figure 2c); FOSB showed a granular cytoplasmic staining mostly limited to

Table 2 Dilution and technique used for each antibody

No.	Antibody	Dilution	Retrieval	Incubation	Amplification	Positive control
1	Prostaglandin D synthase	1:500	Pronase	2 h/room temperature	N/A	Non small cell lung carcinoma
2	CD36	1:100	Microwave	1 h/room temperature	CSA[a]	Prostate carcinoma and normal testis
3	MIP2	1:100	Microwave	1 h/room temperature	N/A	Pancreatic adenocarcinoma and normal liver
4	FOSB	1:500	Microwave	1 h/room temperature	CSA	Breast invasive ductal carcinoma
5	HSP10	1:100	Microwave	4°C overnight	N/A	Colon and uterine cervix carcinomas
6	Pan-cathepsin	1:100	Microwave	1 h/room temperature	N/A	Breast invasive ductal carcinoma and colon adenocarcinoma
7	MMP1	1:250	Microwave	1 h/room temperature	CSA	Breast invasive ductal carcinoma and colon adenocarcinoma
8	GST	1:1	Microwave	1 h/room temperature	N/A	Colon adenocarcinoma
9	Siva	1:10	Pronase	1 h/room temperature	N/A	Colon adenocarcinoma
10	RbAp46	1:500	No	1 h/room temperature	N/A	Breast invasive ductal carcinoma
11	Ki67	1:75	Microwave	1 h/room temperature	N/A	Ovarian serous papillary carcinoma
12	p53	1:500	Microwave	1 h/room temperature	N/A	Ovarian serous papillary carcinoma

[a]Catalyzed signal amplification system (Dako).

cancer cells (Figure 2d); GST had a diffuse cytoplasmic staining limited to cancer cells (Figure 2e); pan-cathepsin was present in both cancer and stromal cells (Figure 2f); prostaglandin D synthetase was present in less than 5% of cancer cells and was not retained for this study; RbAp46 gave a strong positive nuclear staining in basically all cases and was not further analyzed; Siva and MIP2 were completely negative despite repeated attempts with various retrieval systems, antibody concentration, incubating time, or signal enhancements systems and were not retained for the study; p53 (Figure 2g) and Ki-67 (Figure 2h) gave a nuclear staining.

Relation Between Markers and Risk Factors

No association was found between CD36, HSP10 and FOSB and any risk factor. MMP1 overexpression was associated with an older age ($P=0.01$). Overexpression of GST was positively associated with higher initial serum CA15 ($P=0.02$). Pan-cathepsin expression was associated with higher grade ($P=0.02$) and higher initial CA125 levels ($P=0.01$). p53 tended to be associated with an older age ($P=0.06$). High Ki-67 levels ($>20\%$) was associated with higher grade ($0=0.03$). Furthermore, we found no linear correlation between gene expression obtained by microarray and protein expression obtained by immunohistochemistry (data not shown).

Relation Between Marker Expression and Progression-Free Survival

Table 4 shows the prevalence of expression of each marker along with bivariate and multivariate analyses to predict progression-free survival. Multivariate analyses taking into account standard or strongly associated prognostic variables (age, grade, stage, type of chemotherapy, initial CA125) were performed to identify independent prognostic factors. Multivariate analyses showed a significant association between HSP10 expression and a lower risk of progression after chemotherapy (HR: 0.6; CI: 0.42–0.87; $P=0.007$). A Kaplan–Meier curve for HSP10 and progression-free survival is depicted in the Figure 3. High proliferation rate ($Ki67>20\%$) showed a tendency to predict a lower risk of progression post chemotherapy (HR: 0.72; CI: 0.51–1.03; $P=0.07$). A trend was found for MMP1 overexpression to predict a higher risk of progression (HR: 1.61; CI: 0.94–2.79; $P=0.08$).

Discussion

Our study shows that HSP10 is the only significant factor of delayed progression in patients exposed to chemotherapy. These findings confirm those of our micro-array study[12] and are also consistent with the biology of HSP10.

Indeed, heat-shock proteins (HSPs) are important molecules in oncology. Five types of primary HSPs

are currently known, and they are designated according to their molecular weight (HSP27, 60, 70, 90 and 110).[17] HSPs bind and stabilize proteins to prevent the creation of aggregates during protein synthesis, transmembrane transport or stress, such as high temperatures.[18] There are some cochaperones of low molecular weight that often form a biologically active complex. The HSP10-HSP40 complex is known to facilitate interactions between primary HSP and the substrate.[17] The HSP10-HSP60 complex is involved in apoptosis through caspase activation.[19] Therefore, in cases with constitutively low HSP10 levels, chemoresistance, as we observed, may be explained by a lack of capacity to induce apoptosis. However, in another micro-array study, HSP10 mRNA was found to be overexpressed in breast cancer cell cultures exposed to oxyplatin and 5-FU, suggesting that HSP10 overexpression, rather than low expression, might be associated with chemoresistance.[20] However, in such an instance, higher HSP10 levels might not be associated with chemoresistance but might rather be induced by exposure to chemotherapy agents.[18,19]

Table 3 Patients' characteristics

Variable	n/total	%
Age (years)		
<50	27/158	17.1
50–69	89/158	56.3
>70	42/158	26.6
Median age		
61		
Grade		
1	8/158	5.1
2	45/158	28.4
3	105/158	66.5
Stage		
III (A, B and C)	123/158	77.8
IV	35/158	22.2
Second cancer		
Breast	10/158	6.3
Colon	3/158	1.9
Endometrium	1/158	0.6
Skin	2/158	1.3
Lymphoma	1/158	0.6
Type of chemotherapy		
Platinum+taxol	112/158	70.9
Other	46/158	29.1
CA125 initial (U/ml)		
<800	72/158	45.6
>800	69/158	43.6
N/D	17/158	10.8

In addition to the reaction to stress, HSPs play an important role in carcinogenesis. HSP60 and its cochaperone HSP10 are expressed early during the development of a malignant phenotype.[21] In colon and uterine cervix cancers, HSP 10 and 60 expression levels are increased as cells progress from their normal state to dysplasia and cancer.[19,22] HSPs are not expressed only by cancer cells. Indeed, higher levels of HSPs 10 and 60 were found in the cytoplasm of lymphocytes in lymph nodes with metastatic colon cancer than in non-metastatic lymph nodes.[23] The presence of HSP10 was also detected in the serum and ascites from ovarian cancer patients and it was found that HSP10 in the serum may play a role in T lymphocyte inhibition, allowing cancer cells to escape immunitary surveillance.[24]

However, the prognostic significance of HSPs is not clear. In head and neck cancer[25] and in breast cancer,[26] HSP27 was not found to predict survival but HSP27 predicted neck cancer failure after radiation therapy.[27] A few studies investigated the role of HSPs in ovarian cancer. HSP27 was not found to predict response to chemotherapy[5] but HSP

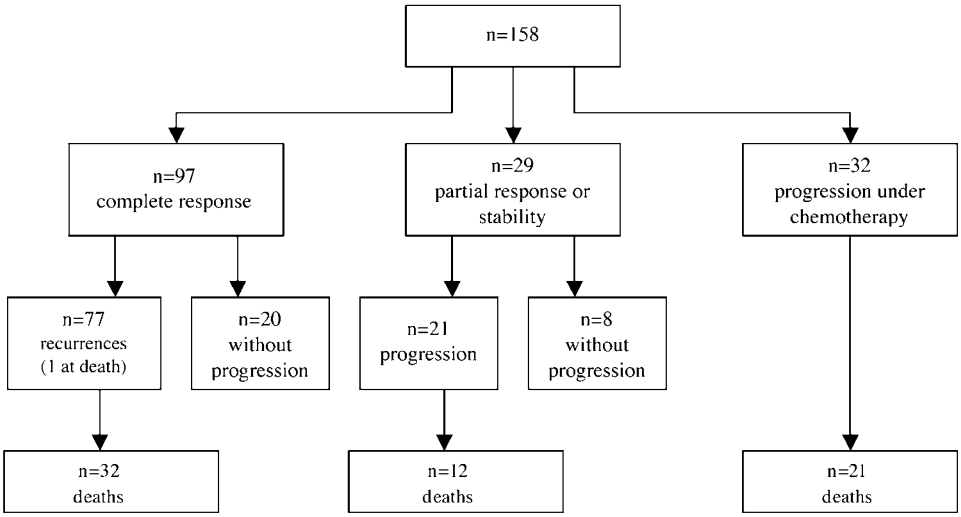

Figure 1 Distribution of patients according to their response to chemotherapy and evolution.

60 overexpression was associated with a poor prognosis.[28]

In our study, Ki-67 failed to significantly predict progression-free survival. However, high proliferation rate was associated with higher tumor grade and Ki-67 tended to predict a better survival without progression, suggesting that high proliferation is associated with chemosensitivity. This is consistent with data from the literature, high proliferation rate, as measured by Ki-67, being a marker of recurrence in soft tissue sarcoma,[29] head and neck cancer[30] and urothelial tumors.[31] It also predicted response to neoadjuvant anthracycline-based chemotherapy in breast cancer[32] and response to radiation therapy in head and neck cancer.[16,33]

In advanced ovarian carcinoma, high Ki-67 index was predictive of recurrence[34] and poor prognosis.[35,36] However, as in our study, low proliferation predicted poor response to chemotherapy in ovarian carcinomas.[37]

In our study, there was a tendency for MMP1 to predict a poorer progression-free survival. MMP1 overexpression by immunohistochemistry was associated with a poor prognosis in both colon[38] and esophageal[39] cancers. However, no such prognostic study has been reported in ovarian cancer. MMP1 expression was found to be negligible in benign cystadenomas but it was overexpressed in both tumor and stromal cells of serous papillary carcinomas.[40] Interestingly, a 2G mutation on the MMP1 promoter was found to be associated with MMP1 overexpression.[41] The authors suggest that such genetic abnormality may be associated with an increased risk of developing ovarian cancer, although others did not reach such a conclusion.[42]

The fact that, of the 12 genes selected by microarrays comparing chemosensitive and chemoresistant tumors, HSP10 was the only significant marker of progression-free survival by immunohistochemistry is intriguing. This may suggest that the 2-fold ratio between high and low expression by microarrays defined in our previous study,[12] is not high enough to be clinically relevant by immunohistochemistry. Furthermore, we found no linear

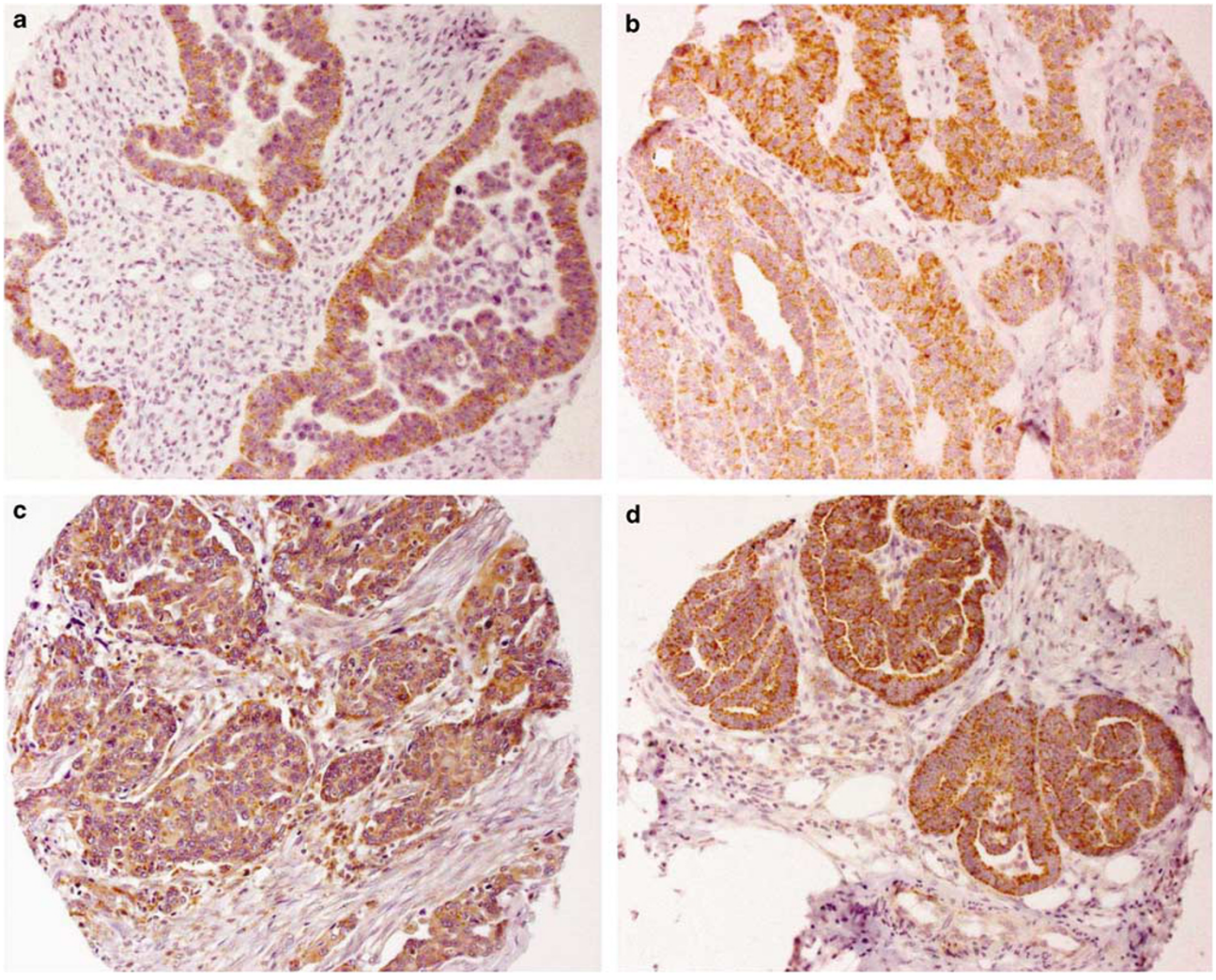

Figure 2 Marker expression by immunohistochemistry: (**a**) MMP1; (**b**) CD36; (**c**) HSP10; (**d**) FOSB; (**e**) GST; (**f**) pan-cathepsin; (**g**) p53; (**h**) Ki-67.

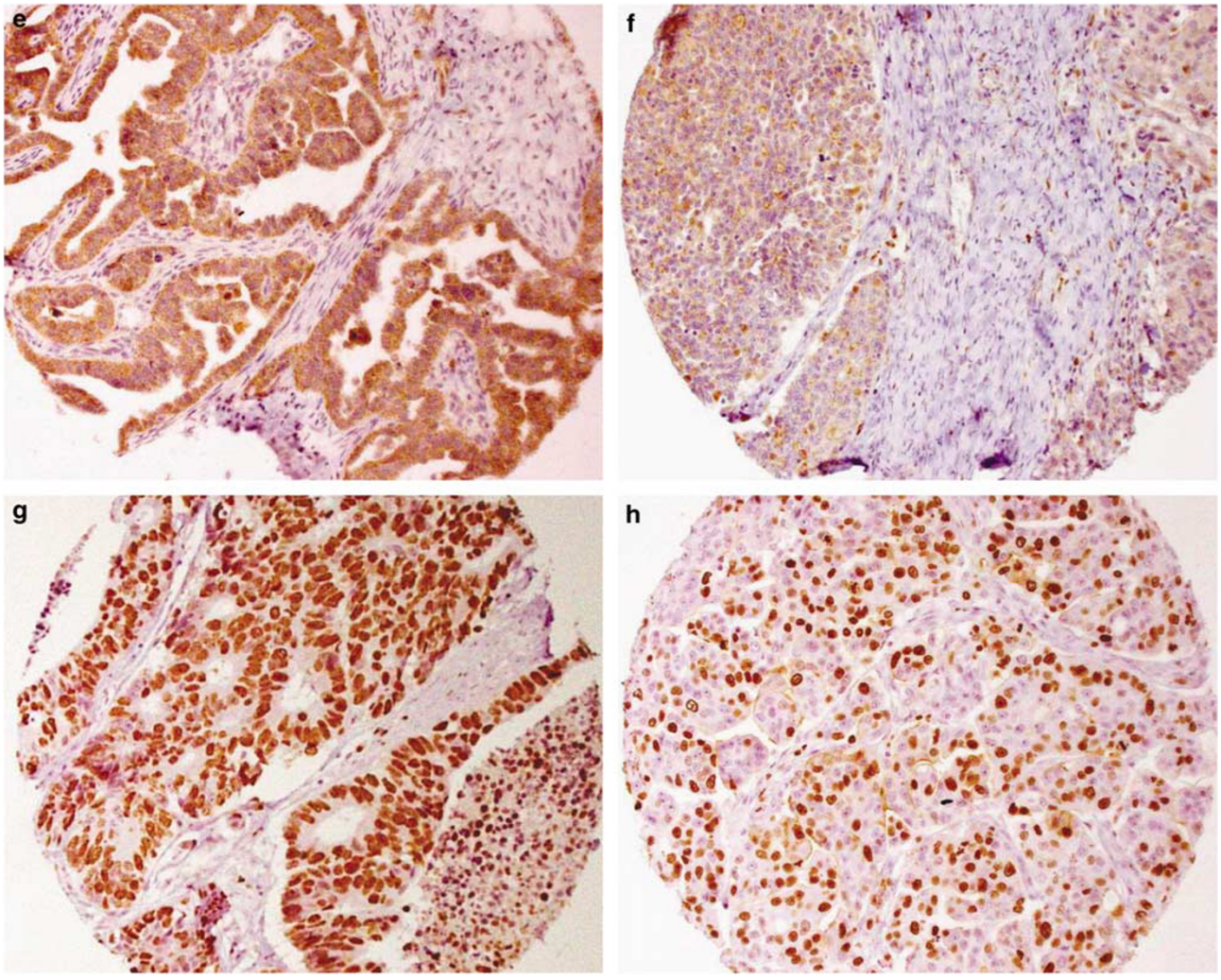

Figure 2 Continued.

Table 4 Cox regression analysis to predict progression-free survival

Marker	Value	Frequency	Event	Crude			Adjusted		
		N (%)	*n* (%)	HR	95% CI	*P*-value	HR[a]	95% CI	*P*-value
CD6	Negative	128 (81.0)	103 (80.5)	1.0			1.0		
	Positive	30 (19.0)	27 (90.0)	1.13	[0.74; 1.73]	0.56	1.055	[0.67; 1.65]	0.81
HSP10	Negative	90 (57.0)	80 (88.9)	1.0			1.0		
	Positive	68 (43.0)	50 (73.5)	0.64	[0.45; 0.91]	0.01	0.60	[0.42; 0.87]	0.007
FOSB	Negative	127 (80.4)	102 (80.3)	1.0			1.0		
	Positive	31 (19.6)	28 (90.3)	1.15	[0.76; 1.76]	0.50	1.07	[0.69; 1.66]	0.77
MMP1	Negative	139 (88.0)	113 (81.3)	1.0			1.0		
	Positive	19 (12.0)	17 (89.5)	1.48	[0.89; 2.47]	0.13	1.61	[0.94; 2.79]	0.08
GST	Negative	116 (73.4)	92 (79.3)	1.0			1.0		
	Positive	42 (26.6)	38 (90.5)	1.25	[0.86; 1.83]	0.25	1.08	[0.72; 1.61]	0.70
Pan-cathepsin	Negative	109 (69.0)	88 (80.7)	1.0			1.0		
	Positive	49 (31.0)	42 (85.7)	0.98	[0.68; 1.42]	0.92	0.92	[0.61; 1.37]	0.67
p53	Negative	67 (42.4)	55 (82.1)	1.0			1.0		
	Positive	91 (57.6)	75 (82.4)	0.815	[0.58; 1.15]	0.24	0.83	[0.58; 1.18]	0.29
Ki67	<20%	83 (52.5)	71 (85.5)	1.0			1.0		
	>20%	75 (47.5)	59 (78.7)	0.76	[0.54; 1.08]	0.13	0.72	[0.51; 1.03]	0.07

[a]Note: adjusted for age, stage, grade, baseline CA125 and type of chemotherapy.

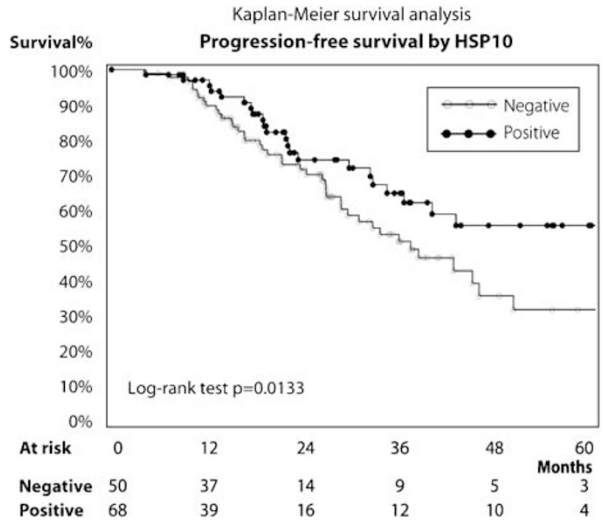

Figure 3 Kaplan–Meier curve for progression-free survival and HSP10.

correlation between gene expression obtained by microarray and protein expression obtained by immunohistochemistry. Current literature also shows that the correlation between mRNA and protein levels is insufficient to predict protein expression levels from quantitative mRNA data. Indeed, for some genes, while mRNA levels are of the same value, the protein levels may vary by more than 20-fold and, conversely, proteins with similar levels may have respective mRNA transcript levels that vary by as much as 30-fold.[43] Further studies should focus on the clinical and immunohistochemistry relevance of data obtained by microarrays.

We conclude that, despite a lack of direct relation between mRNA and proteins levels, gene expression analysis coupled with immunohistochemistry allowed us to identify high HSP10 and possibly, high proliferation and low MMP1 as potential markers of response to chemotherapy. Future studies should be aimed at developing a prognostic index combining the immunohistochemistry markers to predict the response to chemotherapy.

Acknowledgements

This study was financially supported by a grant from the Cancer Research Society of Canada (The Strategic Research Program on Genomics and Proteomics of Metastatic Cancer) and by the Cancer Research Network of the 'Fonds de la Recherche en Santé du Québec', Québec, Canada.

Disclosure/Conflict of Interest

There are no financial conflicts of interest with any organization and for any author of this article.

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