The expression ratio of *Map7/B2M* is prognostic for survival in patients with stage II colon cancer

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Abstract. Colorectal cancer (CRC) is the second most frequent cause of cancer-related death in the United States. To determine whether certain molecular markers might be prognostic for survival, we measured by quantitative real-time RT-PCR the expression levels of 15 previously studied genes that are known to be up-regulated or down-regulated in the progression of epithelial cancers. The tumor samples were extracted from formalin-fixed paraffin-embedded primary tissues derived from patients with Stage II CRC who developed disease recurrence within two years (n=10), or were disease-free for at least 4 years (n=12). We were able to determine, by AUC curve analysis, that the ratio of *microtubule associated protein 7* (*Map7*)/*B2M* was predictive of outcome in our sample set. Further, using Kaplan-Meier survival analysis, we observed significantly different curves as a function of marker positivity for the *Map7/B2M* (p=0.0001; HR=11) expression ratio. This suggests that the expression ratio of *Map7/B2M* may serve as a valuable prognostic marker in patients with Stage II colon cancer, and potentially guide therapeutic decision making.

Introduction

Colorectal cancer (CRC) is the fourth most common non-cutaneous malignancy in the United States and the second most frequent cause of cancer-related death. In 2007, an estimated 153,760 cases of colorectal cancer were expected to be diagnosed, with 52,180 deaths expected from the disease (1). Clinically, the most important determinant of colon cancer survival is stage. The tumor-node-metastasis (TNM) system, as defined by the American Joint Committee on Cancer (AJCC), is the most commonly used cancer staging system and classifies colon cancer into four stages based on depth of invasion of the bowel wall (T), extent of regional lymph node involvement (N), and presence of distant sites of metastatic disease (M) (2). Stage I includes T1 and T2 tumors without nodal or distant metastases and most patients with this disease are cured with segmental colectomy alone. The overall 5 year survival (OS) of this stage is 93.2%. Stage II is subdivided into two classes (IIA and IIB; OS=84.7 and 72.2%) and includes T3 and T4 tumors, respectively. Like Stage I, metastases are absent in Stage II disease. Although many patients with Stage II disease are cured by surgical resection, between 20 and 40% of patients with completely resected Stage II disease do not survive 5 years (3,4).

Stage III disease includes tumors with evidence of nodal disease but without distant metastases. After complete surgical resection, these patients face a 50-60% chance of developing recurrent disease. However, adjuvant therapy with 5-fluorouracil based regimens has been shown to improve overall survival by roughly 30% (5). As a result, the NIH issued a consensus statement that recommended chemotherapy as an adjuvant to surgery in all medically fit patients with Stage III colon cancer (6).

The benefit of adjuvant therapy in surgically resected Stage II patients has been less clear and remains a subject of debate. In response, the American Society of Clinical Oncology (ASCO) recently issued a statement discouraging the routine administration of adjuvant therapy in patients with Stage II disease, largely due to the fact that no single randomized clinical trial has demonstrated a survival benefit for such patients (7). Due to the controversy surrounding treatment of Stage II patients, it would be useful to identify

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Table I. Primer sequences used for gene-specific reverse transcription and for real-time RT-PCR.

Gene name	Accession number	Sequence 5' to 3'[a]	Amplicon length (bp)
AGR2/XAG	NM_006408	GCAGAGCAGTTTGTCCTCCTCA GGACATACTGGCCATCAGGAGA	76
B2M	NM_004048	GCCGTGTGAACCATGTGACTTT CCAAATGCGGCATCTTCAAA	97
CEA6	NM_002483	AGATTGCATGTCCCCTGGAA CATTGAATGGCGTGGATTCA	104
CK19	NM_002276	AACGGCGAGCTAGAGGTGA TTCCGTCTCAAACTTGGTTCG	204
Claudin7	NM_001307	TGGCCATCAGATTGTCACAGAC CCAGCCAATAAAGATGGCAGG	88
E-Cadherin/CDH1	NM_004360	CCCACCACGTACAAGGGTC CTGGGGTATTGGGGGCATC	94
Elf3/Esx/Ese-1	NM_004433	TCTTCCCCAGCGATGGTTTT TTGCTCTTCTTGCCCTCGA	124
EpCAM1/TACSTD1	NM_002354	CGCAGCTCAGGAAGAATGTG TGAAGTACACTGGCATTGACGA	88
EpCAM2/TACSTD2	NM_002353	ACCCGAGGAGAAGAGGAGTTTG GCTTCTTTCCCAGTGACAAGCA	100
GPX2	NM_002083	GGACATCAGGAGAACTGTCAGA GTCCTTCAGGTAGGCGAAGAC	150
MAL2	NM_052886	GTCTGCCTGGAGATTCTGTTCG TCACGGACACAAACATGACCC	103
MAP7	NM_003980	AGGACAAAGAACGCCACGAA CACGACCAACGGTTATGCTTC	87
P-Cadherin/CDH3	NM_001793	TCATCGTGACCGACCAGAAT GGATGGAGTAAGCAACCACCC	166
S100P	NM_005980	GACGTCTTTCCCGATATTCGG CCACGGCATCCTTGTCTTTTC	127
Spint2	NM_021102	GTGCCTCAAGAAATGTGCCACT ACAGAGGAATCCGCTGCATTC	81

[a]Upper sequence represents forward primer; lower represents reverse. Underlined sequence represents primer used for reverse transcription.

which patients are at high risk of recurrence and therefore more likely to benefit from the administration of adjuvant chemotherapy (8).

A molecular assay based on the expression profiles of primary tumors can predict patient outcome and prove useful in directing therapeutic interventions (9). One potential assay involves cDNA microarray technology that measures the mRNA expression of thousands of genes using oligonucleotide probes (10). Eschrich *et al* used a 32,000 gene cDNA microarray and 78 human colon cancer specimens (Stages I-IV) and identified a biomarker panel of 43 core genes that predicted the 36-month OS with 90% accuracy (11). Wang *et al* used a 22,000 gene cDNA microarray from 74 patients with Stage II colon cancer and identified a 23-gene signature that predicted OS in 36 patients with 78% accuracy (12). Notably, the gene signatures identified by the two groups had no genes in common. This lack of congruency, in combination with the requirement that the tissues for cDNA microarray analysis must be fresh or frozen, highlights problems applying this technology in the clinic. Determining the expression ratios of just two genes from readily available paraffin-embedded tissue would be less costly, less time consuming and more practical for clinical application.

In this study, we present a novel approach for the development of a prognostic real-time RT-PCR assay in formalin-fixed paraffin-embedded (FFPE) primary tissues. We suggest that the clinical outcome of patients with Stage II colon cancer can be predicted by measuring the expression ratio of *microtubule associated protein 7* (*Map7*)/*B2M* which could allow clinicians to tailor chemotherapeutic treatment to the patient's individual risk of recurrence.

Materials and methods

Patients and tissues. This study was approved by the Institutional Review Board, Medical University of South Carolina.

Table II. Patient characteristics.

Variable	Recurrence		p-value
Age			
<80	5/15	(33%)	0.17
≥80	5/7	(71%)	
Gender			
Male	7/14	(50%)	0.68
Female	3/8	(38%)	
Site			
Cecum	1/4	(25%)	N/C[a]
Ascending colon	2/5	(40%)	
Hepatic flexure	2/3	(67%)	
Transverse colon	3/5	(60%)	
Splenic flexure	0/1	(0%)	
Sigmoid	2/4	(50%)	
Type			
Adenoca	7/18	(39%)	0.29
Adenoca, mucinous	3/4	(75%)	
Size			
≤5	6/11	(55%)	0.37[b]
>5	2/7	(29%)	
Unknown	2/4	(50%)	

[a]Not calculated due to sparseness of data. [b]Data from 'unknown' was not included in calculation of p-value.

Primary tumor specimens. We analyzed primary tumors from Stage II patients who developed disease recurrence within 2 years (n=10), or who lived disease-free for at least 4 years (n=12). Duplicate 50-μm sections were cut for real-time RT-PCR studies and an additional 5-μM section was used for hematoxylin and eosin (H&E) staining.

RNA isolation from paraffin sections. RNA extraction followed the method of Specht *et al* (23) Briefly, paraffin-embedded tissue sections were deparaffinized twice with 1 ml of xylene at 37˚C or room temperature for 10 min. The pellet was subsequently washed with 1 ml of 100, 90, and 70% ethanol and air-dried at room temperature for 2 h. The pellet was re-suspended in 200 μl of RNA lysis buffer (2% lauryl sulfate, 10 mmol/l Tris-HCl pH 8.0, and 0.1 mmol/l EDTA) and 100 μg of proteinase K and incubated at 60˚C for 16 h. RNA was extracted using 1 ml of phenol/chloroform (5:1) solution (Sigma, St. Louis, MO). The aqueous layer containing RNA was transferred to a new 1.5-ml tube. Phenol/chloroform extraction was done a total of three times. RNA was precipitated with an equal volume of isopropanol, 0.1 volume of 3 mol/l sodium acetate, and 100 μg of glycogen at -20˚C for 16 h. After centrifugation at 12,000 rpm for 15 min (4˚C), the RNA pellet was washed with 70% ethanol and air-dried at room temperature for 2 h. Finally, the pellet was dissolved in 12 μl of DEPC water and treated with DNAse prior to cDNA synthesis.

cDNA synthesis and real-time RT-PCR. Complementary DNA (cDNA) was made from 6 μl of RNA described above, 200 U of M-MLV reverse transcriptase (Promega, Madison, WI) and a panel of truncated gene-specific primers (see Table I). Real-time RT-PCR was performed using a PE Biosystems Gene Amp® 7300 or 7500 Sequence Detection System (Foster City, CA). With the exception of the SYBR Green I master mix (Qiagen, Valencia, CA), all reaction components were purchased from PE Biosystems. Standard reaction volumes were 10 μl and contained 1X SYBR RT-PCR buffer, 3 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, 0.4 mM dUTP, 0.1 U UngErase enzyme, 0.25 U AmpliTaq Gold, 0.35 μl cDNA as a template, and 50 nM of oligonucleotide primers. Initial steps of RT-PCR were 2 min at 50˚C for the activation of UngErase, followed by a 10-min hold at 95˚C. Cycles (n=40) consisted of a 15 sec melt at 95˚C, followed by a 1 min annealing/extension at 60˚C. The final step was a 60˚C incubation for 1 min. All reactions were performed in triplicate. Prior to cDNA synthesis, RNA was treated with or without DNAse as described in the text.

Gene expression and statistical analysis. To quantitate gene expression the ΔΔCt method was used. As an internal reference, we used the mean Ct value of all genes. Area under the curve analysis (AUC) for Receiver Operating Characteristics (ROC), was used to measure predictive ability of gene ratios to differentiate patient groups. Kaplan-Meier survival curves were created to look for differences in survival by expression groups and differences between survival curves were quantified and tested using Cox regression models. Statistical analysis was performed using MedCalc software (Mariakerke, Belgium).

Results

Low expression of B2M and high expression of microtubule associated protein 7 (Map7) is a prognostic indicator of survival in Stage II patients. To identify a gene ratio that was predictive of outcome, RNA was isolated from FFPE primary tumor sections from Stage II patients (Table II) who developed disease recurrence within 2 years (n=10), or were disease-free for at least 4 years (n=12) and analyzed for the expression of 15 carcinoma-associated genes listed in Table I. cDNA synthesis and RT-PCR were performed on the purified RNA in duplicate. PCR values were then averaged and AUC analysis was performed by using the mean of all 15 genes as an internal reference control. We observed that the *B2M* expression level was prognostic for disease recurrence (AUC=0.80, 95% CI=0.59-0.94; see also Fig. 1). Low *B2M* expression was associated with disease recurrence, and therefore, poor prognosis.

Having obtained evidence that down-regulation of *B2M* was an independent prognostic factor, we next paired this gene with each of the remaining genes (*gene X*/*B2M*) to determine if a two-gene expression ratio would be at least as prognostic as *B2M* versus the entire gene set. We observed that the expression ratios of *Map7*/*B2M* held higher prognostic value than the *B2M* expression level alone (AUC=0.94, 95% CI=0.75-0.99).

To determine if any other expression ratios might outperform *Map7*/*B2M*, we systematically tested all remaining two-gene combinations (n=78) (*gene X*/*gene Y*) and found

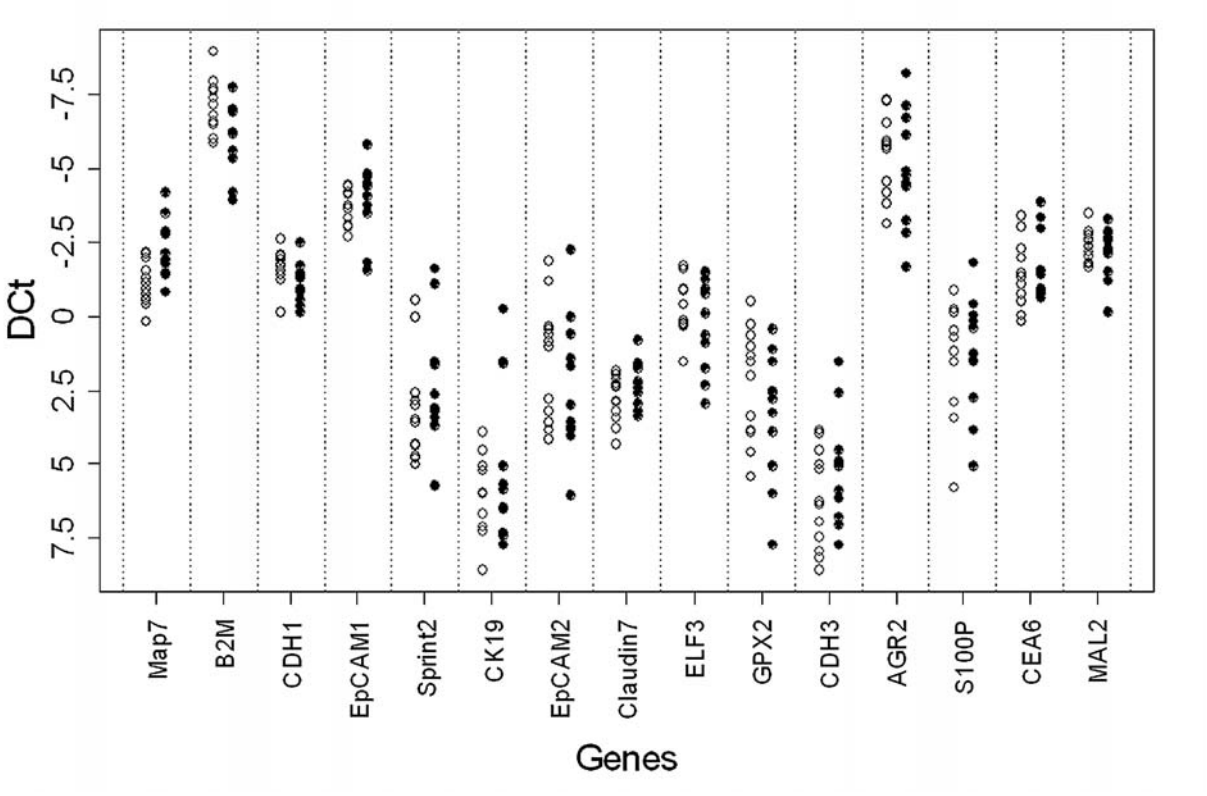

Figure 1. Real-time PCR analyses of formalin fixed paraffin-embedded primary tissue from Stage II colorectal cancer patients. Real-time RT-PCR was performed on tissue samples from patients without (n=12; left side of each matched data set; open circles), and with (n=10; right side of each matched data set; filled circles) develop disease recurrence within two years as described in Materials and methods using primer pairs for the indicated genes. Ct values for each gene were determined from triplicate reactions.

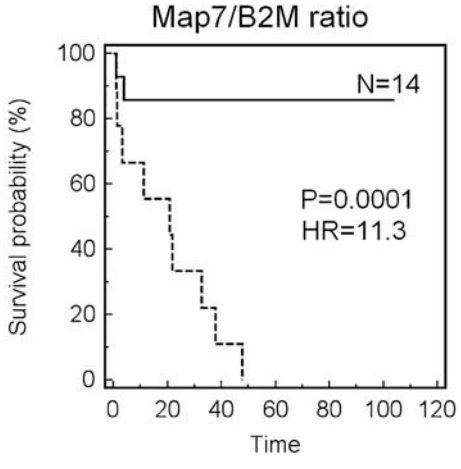

Figure 2. Kaplan-Meier survival analysis of Stage II colon cancer using *Map7*/*B2M* expression ratio as a prognostic factor. Patients were dichotomized according to the *Map7*/*B2M* expression ratio and assigned marker status accordingly. Those patients with higher values and therefore lower *Map7* to *B2M* expression were labeled marker negative (n=14) as those with lower ratio values and therefore higher *Map7* to *B2M* expression were labeled marker positive (n=8). This demonstrated that the *Map7*/*B2M* ratio was able to accurately predict patient survival in our data set.

that this ratio was among the best. The only other ratio that performed nearly as well was *EpCAM1*/*B2M* (AUC=0.85, 95% CI=0.64-0.96). When calculated with respect to the 15-gene set, the AUC value of *EpCAM1* was 0.66 (95% CI=0.43-0.84), providing evidence that this gene was either a weak prognostic factor and/or was serving as a reference control gene.

To determine if *Map7* was simply acting as a reference gene or, alternatively, if it was acting as an independent prognostic marker, we calculated the AUC value of *Map7* with the 15-gene reference set. We observed that *Map7* held the highest independent prognostic value (AUC=0.81, 95% CI=0.59-0.94) of all 15 markers, such that its up-regulation was associated with tumor recurrence and poor prognosis in our patient set.

We next sorted all samples by the value of the *Map7*/*B2M* ratio in descending order and discovered an obvious dichotomy with respect to outcome. The large majority of samples from patients with good outcomes fell near the top of the listing (where the *Map7*/*B2M* ratio held a higher ΔCt value and therefore showed lower expression of *Map7* to *B2M*) and the majority of samples from patient with poor outcomes fell near the bottom (where the *Map7*/*B2M* ratio held a lower value and therefore showed higher expression of *Map7* to *B2M*). A line of division was drawn such that it best separated the samples with respect to outcome. We then labeled each sample above the line as 'marker negative' and each sample below as 'marker positive' and a Kaplan-Meier survival curve was constructed. This yielded highly significant curves comparing marker status and outcome (*Map7*/*B2M*: p=0.0001, HR=11.3; see Fig. 2).

Discussion

In this study, we observed that the ratio of *Map7*/*B2M* was predictive of outcome in Stage II colon cancer patients. Two-gene expression ratios have been previously described as potential guides to therapeutic decision-making in cancer patients. The two-gene expression ratio *HOXb13*/*IL17BR*, was shown to accurately predict tumor recurrence in a subset of ER+ breast cancer patients treated with adjuvant tamoxifen monotherapy (8). In a follow-up investigation using RT-PCR to evaluate the expression profiles of *HOXB13* and *IL-17BR* in ER+ breast cancer samples (n=206), the ratio of these two genes was demonstrated to be predictive of disease relapse in node-negative patients (13). The prognostic accuracy of the *HOXB13*/*IL-17BR* ratio was comparable to that of the 21-gene set that makes up the Oncotype Dx assay which is widely used in clinical practice (14,15). This suggests the possibility that a two-gene prognostic assay may eventually find a place in clinical decision making.

In the present study, we identified that the two-gene expression ratio of *Map7*/*B2M* yielded an HR of 11 for clinical outcome in patients with Stage II disease. High expression of *Map7* was associated with poor prognosis, whereas low expression of *B2M* was associated with poor prognosis. The mechanism by which down-regulation of *B2M* expression might contribute to tumor progression is not well understood. *B2M* is a chaperone of the major histocompatibility complex (MHC) class I, and MHC1-like, molecules that play a central role in antigen presentation and immunoglobulin transport. In the tumor host immune response, HLA-A,B,C assembles with *B2M* at the cell surface (16,17). Loss of these class 1 antigens is associated with decreased histological differentiation in colon cancer (18), as well as increased malignancy in a number of neoplasms, including B cell lymphoma and melanoma (16,17). Interestingly, loss of the native HLA-A,B,C/*B2M* complex appears to be sporadic in nature; in some cases the loss is localized to certain portions of the tumor, whereas in others, loss of *B2M* is evident across the entire tumor (16). Since MHC class 1 antigens are required for the host to mount a tumor response, the loss of these antigens may allow a tumor to escape recognition by the immune system.

There is little known about the role of *Map7* in cancer progression. As a family, microtubules are involved in many important cellular processes including cell division, motility, and changes in cell shape where MAPs bind to and stabilize microtubules (19). Chemotherapeutic agents, such as Paclitaxel, act by altering microtubule-associated mitotic processes ultimately leading to cell apoptosis. *Map7* was first described by Masson in 1993 as being involved in microtubule stabilization and epithelial cell differentiation (20). During prophase, *Map7* is hyperphosphorylated and inactive; it is activated by dephosphorylation and then slowly reassociates with microtubules. Komada *et al* determined that *Map7* is involved in microtubule organization of both Sertoli cells and spermatids in the seminiferous tubules (21), events that require cell migration. *Map7* is also highly expressed at the floor of the neural plate, where migration commences to form the central and peripheral nervous systems (21). In support of a potential role of *Map7* in metastatic growth, this gene was recently identified as one out of only fifteen that was highly up-regulated in metastatic endometrial cancer using a 22K Affymetrix array (22). Additional studies are necessary to elucidate the roles, interations, and functions of the *Map7* and *B2M* genes during colorectal cancer tumor progression. We have recently begun studies to determine what effect, if any, up-regulation/down-regulation of *Map7* may have on cell motility, proliferation, and/or invasive potential. We are also working to obtain tissue from additional patients with Stage II colon cancer with sufficient follow-up data such that the ratio may be validated in a larger patient set.

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References

1. Greene FL, Stewart AK and Norton HJ: A new TNM staging strategy for node-positive (stage III) colon cancer: an analysis of 50,042 patients. Ann Surg 236: 416-421, 2002.
2. Morris M, Platell C, McCaul K, *et al*: Survival rates for stage II colon cancer patients treated with or without chemotherapy in a population-based setting. Int J Colorectal Dis 22: 887-895, 2007.
3. Macdonald JS: Adjuvant therapy of colon cancer. CA Cancer J Clin 49: 202-219, 1999.
4. Moertel CG, Fleming TR, Macdonald JS, *et al*: Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 322: 352-358, 1990.
5. NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. JAMA 264: 1444-1450, 1990.
6. Wolpin BM, Meyerhardt JA, Mamon HJ and Mayer RJ: Adjuvant treatment of colorectal cancer. CA Cancer J Clin 57: 168-185, 2007.
7. Macdonald JS and Haller D: Update on adjuvant therapy of colon cancer. Tumori 83: S39-S41, 1997.
8. Ma XJ, Wang Z, Ryan PD, *et al*: A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell 5: 607-616, 2004.
9. Hegde P, Qi R, Abernathy K, *et al*: A concise guide to cDNA microarray analysis. Biotechniques 29: 548-562, 2000.
10. Eschrich S, Yang I, Bloom G, *et al*: Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 23: 3526-3535, 2005.
11. Wang Y, Jatkoe T, Zhang Y, *et al*: Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol 22: 1564-1571, 2004.
12. Shah SA, Simpson SJ, Brown LF, *et al*: Development of colonic adenocarcinomas in a mouse model of ulcerative colitis. Inflamm Bowel Dis 4: 196-202, 1998.
13. Goetz MP, Suman VJ, Ingle JN, *et al*: A two-gene expression ratio of homeobox 13 and interleukin-17B receptor for prediction of recurrence and survival in women receiving adjuvant tamoxifen. Clin Cancer Res 12: 2080-2087, 2006.
14. Paik S, Shak S, Tang G, *et al*: A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 351: 2817-2826, 2004.
15. Paik S, Shak S, Tang G, *et al*: Development and validation of a multi-gene RT-PCR assay for predicting recurrence in node negative, ER+, tamoxifen-treated breast cancer patients: NSABP Studies B-20 and B-14. San Antonio Breast Cancer Symposium, 2004.
16. Momburg F and Koch S: Selective loss of beta 2-microglobulin mRNA in human colon carcinoma. J Exp Med 169: 309-314, 1989.
17. Momburg F, Ziegler A, Harpprecht J, *et al*: Selective loss of HLA-A or HLA-B antigen expression in colon carcinoma. J Immunol 142: 352-358, 1989.
18. Gattoni-Celli S, Kirsch K, Timpane R and Isselbacher KJ: Beta 2-microglobulin gene is mutated in a human colon cancer cell line (HCT) deficient in the expression of HLA class I antigens on the cell surface. Cancer Res 52: 1201-1204, 1992.

19. Bhat KM and Setaluri V: Microtubule-associated proteins as targets in cancer chemotherapy. Clin Cancer Res 13: 2849-2854, 2007.
20. Masson D and Kreis TE: Binding of E-MAP-115 to microtubules is regulated by cell cycle-dependent phosphorylation. J Cell Biol 131: 1015-1024, 1995.
21. Komada M, McLean DJ, Griswold MD, Russell LD and Soriano P: E-MAP-115, encoding a microtubule-associated protein, is a retinoic acid-inducible gene required for spermatogenesis. Genes Dev 14: 1332-1342, 2000.
22. Ferguson SE, Olshen AB, Viale A, *et al*: Gene expression profiling of tamoxifen-associated uterine cancers: evidence for two molecular classes of endometrial carcinoma. Gynecol Oncol 92: 719-725, 2004.
23. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H: Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 158: 419-429, 2001.