Short Communication

Differential Expression of a New Tumor-Associated Antigen, TLP, During Human Colorectal Cancer Tumorigenesis

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Tumour liberated particles (TLP) have been proposed as a potential new serum tumor marker. In particular, a high percentage of patients with early stages of lung cancer scored positive for serum TLP, suggesting its possible role as a marker for early diagnosis of disease. The aim of the present study was to analyze the expression of TLP in the colorectal adenoma-carcinoma sequence in order to determine whether its expression correlates with the various stages of cancer transformation. TLP distribution was assessed by immunohistochemistry in normal, premalignant, and malignant colorectal lesions. Normal colonic mucosa and hyperplastic polyps showed no positive staining, whereas adenomas and adenocarcinomas reacted to anti-TLP serum. The percentage of positive tumor cells increased from adenomas with mild dysplasia to adenomas with severe dysplasia. Moreover, a supranuclear staining pattern was observed mainly in adenomas with mild dysplasia, whereas adenomas with severe dysplasia as well as adenocarcinomas showed a characteristic diffuse staining pattern and a strong staining intensity. Only a few cases of adenocarcinoma were found to be TLP-negative and all were poorly differentiated. Our results suggest that TLP antigen expression may be considered as a marker of epithelial atypia in the colorectal tract and as a potential target for new diagnostic and/or therapeutic approaches to human colorectal cancer. (*Am J Pathol* 1999, 154:993-999)

One of the major areas of interest in current research in oncology is the evaluation of phenotypic alterations that might help to define groups of patients whose lesions are more likely to undergo malignant transformation. To date, several tumor antigens, ie, carcinoembryonic antigen (CEA), tumor-associated glycoprotein-72 (TAG-72), and blood group-related antigens, have been associated with parameters conventionally believed to indicate malignant potential, such as degree of dysplasia.¹⁻⁶ Moreover, extensive studies have shown the clinical utility of the measurement of serum expression of these antigens as an additional diagnostic parameter for early diagnosis of recurrent disease during postsurgical follow-up.^{1,7-13}

In the past 15 years, several new tumor-associated antigens have been identified and characterized. Among those, tumor liberated particles, also referred as tumor liberated protein (TLP) by other authors have recently been proposed as a new tumor-associated antigen. First described by Tarro in 1983,¹⁴ TLP is a complex of proteins showing the characteristics of glycoproteins, which were found in various types of human cancer. One of the major components of this complex was isolated from lung tumor tissues and identified as a 214-kd glycoprotein.^{14,15} Subsequent protein-sequencing studies identified in a 100-kd fragment of this protein, a nonapeptide epitope (RTNKEASIC) termed CSH-275, which was used to obtain a rabbit anti-TLP serum.¹⁶ Meanwhile, a study performed by Garaci and coworkers¹⁷ suggested that this molecule may potentially be a new serum tumor marker for lung cancer. In particular, a high percentage of patients with early stages of malignant disease scored positive for serum TLP, suggesting its possible role as a marker for an early diagnosis of lung cancer.¹⁷ Therefore, the aim of the present study was to analyze the expression of TLP in the colorectal adenoma-carcinoma sequence, which represents one of the best known models

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of human cancer transformation. For this purpose, TLP expression was assessed in normal, premalignant, and malignant colorectal lesions to determine whether its expression could be considered an early marker of cancer transformation.

Materials and Methods

Tissue Samples

Tissue samples were obtained from 85 patients with malignant ($n = 43$) or benign ($n = 42$) colorectal diseases, surgically resected at "Regina Elena" Cancer Institute and University of "Tor Vergata" (Rome, Italy). Specimens of normal, histologically confirmed colonic mucosa from 21 carcinoma patients were also obtained. In addition, tissue samples of normal colonic mucosa from 19 healthy donors enrolled in an institutional program of surveillance for colorectal cancer were analyzed. Polyps and colorectal carcinomas were classified according to World Health Organization criteria (Table 1).^{18,19} Adenomas were graded by histologic and cytologic features into three grades of epithelial dysplasia: mild, moderate, and severe, according to the criteria proposed by Konishi and Morson.²⁰

Immunization Protocol and Characterization of Rabbit Anti-TLP Serum

Rabbit anti-TLP serum (obtained at Cold Spring Harbor Laboratory and kindly provided by Istituto Farmacoterapico Italiano SpA, Rome, Italy) was obtained by immunizing four rabbits subcutaneously with 0.5 mg of antigen in 0.5 ml of phosphate buffered saline (PBS), mixed with 0.5 ml of Freund's complete adjuvant. Booster injections with incomplete adjuvant were given at 2-week intervals. Sera were collected on alternate weeks *via* ear vein bleeds. Titers of antisera were performed by radioimmunoassay on 96-well microtiter plates. Wells were coated with a fixed concentration of peptide antigen, washed, and incubated with various dilutions of sera. The bound immunoglobulin was detected with ¹²⁵I-labeled protein A and quantified by radioimmunoassay for the four rabbits. At a dilution of 1:1,000, all sera showed a bound radioactivity of 15,000 cpm *versus* a background of 1,000–1,500 cpm, indicating a positive staining reaction with the peptide antigen (data not shown). Preimmune sera from the corresponding rats were also tested and no significant reactivity was detected.

Western Blot Analysis

Whole-protein extracts from colorectal carcinoma tissue biopsies were obtained as previously reported.²¹ Samples containing 30 μ g of proteins, measured by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) were analyzed using a 5–20% linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were elec-

trophoretically transferred to a 0.45- μ m nitrocellulose membrane (Bio-Rad Laboratories) overnight at 4°C. The membranes were washed in a blocking buffer and incubated overnight at 4°C in 1:400 dilution of the rabbit anti-TLP serum, the preimmune serum and with an anti-CEA mouse monoclonal antibody, COL-1, at 1 μ g/ml (kindly provided by Dr. J. Schlom, National Cancer Institute, National Institutes of Health, Bethesda, MD).²² After washing with PBS containing 0.05% Tween-20, the blots were incubated 1 hour with a 1:2000 dilution of the second appropriate horseradish peroxidase-conjugated antibody. Following three washes with PBS-Tween-20, bands were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL) and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham). Preimmune sera from the corresponding rats were also tested and did not show a significant reactivity.

Immunohistochemical Analysis

Sections (5 μ m) from each formalin-fixed paraffin-embedded tissue block were cut and mounted on slides coated with APES (Dako, Glostrup, Denmark). TLP antigen expression was evaluated using the avidin-biotin complex method as previously described.²³ Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanols, and treated with methanol containing 0.3% H₂O₂ to block endogenous peroxidase activity. After rinsing in PBS without Ca²⁺ and Mg²⁺, pH 7.4, slides were incubated in 10% normal horse serum (NHS) for 30 minutes at room temperature. This latter and all subsequent reagents were diluted in PBS containing 0.1% bovine serum albumin (BSA), added at 200 μ l/slide and incubated in a sealed moisture chamber. NHS was removed and slides were incubated overnight at 4°C with rabbit anti-TLP or the control pre-immune serum at a 1:400 dilution. In addition, a positive control antibody (mouse anti-cytokeratin mAb, Immunon, Pittsburgh, PA) and an isotype matched mouse monoclonal antibody (MOPC 21, Sigma, Chemical Co., St. Louis, MO) were used to verify accurate staining method. Primary Abs were removed, and slides were washed twice in PBS. Horse anti-rabbit or anti-mouse IgG immunoglobulin (Vector Laboratories, Burlingame, CA) were used at 1:100 or 1:500 dilutions, respectively. After an incubation of 30 minutes and two washes in PBS, avidin-biotin complex (Vector Laboratories) was added for 30 minutes at room temperature. The slides were rinsed in PBS and the peroxidase reaction was initiated using 0.06% diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. After a final PBS rinse, the sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted under a coverslip using Permount (Fisher Scientific, Fair Lawn, NJ).

Scoring Method

Each section was evaluated for the presence of cell-associated (cytoplasmic or membranous) as well as ex-

Figure 1. Immunodetection by Western blot analysis of TLP expression in whole-protein extract obtained from a colorectal carcinoma tissue biopsy using the rabbit anti-TLP serum (lane 1), the preimmune serum from the same rabbit (lane 2) and a positive control mouse monoclonal antibody anti-CEA (COL 1) (lane 3).

tracellular diaminobenzidine precipitate (reddish-brown), indicative of primary Ab binding. The intensity of staining was graded as 0 (negative), +, ++, or +++. In addition, staining compartmentalization was categorized as either supranuclear (immunoreactivity localized exclusively in apical cytoplasmic area) or diffuse (immunoreactivity present in the whole cytoplasm). Faint blushes ($\pm$) were considered negative, and any reactivity not dark enough to be identified using $\times 40$ magnification was considered a blush. Furthermore, the approximate percentage of positive cells for each malignant tissue was scored ac-

cording to the number of positive malignant cells divided by the total number of malignant cells $\times 100$. The approximate percentage of positive cells for benign tumors was scored according to the number of positive benign epithelial cells divided by the total number of benign epithelial cells $\times 100$. For normal tissues, the percentage of reactivity is noted for each cell type and represents an approximation of the number of positive cells divided by the total number of cells of that type $\times 100$. Staining was recorded as negative if $\leq 5\%$ of the tumor or epithelial cells were positive.

Results

Figure 1 shows the specific reactivity of the serum from the immunized rabbit versus a molecule of 100 kd present in the protein extract obtained from a human colorectal tissue biopsy (lane 1), while the preimmune serum from the same animal did not show any reactivity (lane 2). A mouse monoclonal antibody (COL 1) recognizing the CEA molecule has been used as a positive control (lane 3).

The presence of TLP antigen was analyzed on serial sections of formalin-fixed, paraffin-embedded malignant, benign, and normal colonic tissues, using a rabbit anti-TLP serum by means of immunohistochemistry. Optimal assay conditions were found using the rabbit anti-TLP serum at 1:400 dilution and overnight incubation (16–18 hours) at 4°C , which increased the percentage of cellular reactivity as well as assay sensitivity and decreased the background staining.

Normal Colonic Mucosa from Healthy Donors

Tissue biopsies of normal colonic mucosa from 19 healthy volunteers were studied. On microscopic examination only mild chronic inflammatory infiltration associated with edema of the submucosa was observed. As reported in Table 1, none of the 19 cases showed a positive staining. An example of the immunohistochemical analysis of a normal colonic mucosa is shown in Figure 2A.

Table 1. TLP Antigen Expression in Normal, Benign, and Malignant Tissues (Correlation with Degree of Dysplasia or Degree of Differentiation in Neoplastic Lesions)

Tissue specimens	No. of positive tissues/No. of tissues studied (%)	% positive cells $\pm$ SEM	Staining compartmentalization	
			Diffuse (%)	Supranuclear (%)
Normal colonic mucosa	0/19 (0.0%)	N/A	N/A	N/A
Hyperplastic polyps	0/10 (0.0%)	N/A	N/A	N/A
Adenomas				
Mild dysplasia	9/9 (100.0%)	33.0 $\pm$ 1.6	22.2	77.8
Moderate dysplasia	15/16 (93.7%)	50.0 $\pm$ 3.2	62.5	37.5
Severe dysplasia	7/7 (100.0%)	85.1 $\pm$ 1.8	>95.0	0.0
Adenocarcinomas				
Well differentiated	7/7 (100.0%)	88.3 $\pm$ 2.2	>95.0	0.0
Moderately differentiated	25/25 (100.0%)	76.2 $\pm$ 3.0	>95.0	0.0
Poorly differentiated	6/11 (54.5%)	45.9 $\pm$ 4.4	>95.0	0.0
Corresponding normal mucosa	8/21 (38.0%)	10.6 $\pm$ 3.8	0.0	>95.0

Values represent the mean of results obtained in all positive cases. SEM, standard error of the mean; N/A, not applicable.

Figure 2. Immunohistochemical evaluation of TLP expression in normal colonic mucosa obtained from a normal donor (A), of an inflammatory polyp (B), and on a tubulo-villous adenoma with moderate dysplasia (D), and its correspondent normal mucosa at the base of the lesion (C). All panels were hematoxylin-counterstained. Magnifications, $\times 100$ (A, C, and D) and $\times 200$ (B).

Colorectal Polyps

The resected polyps included 42 colorectal lesions (10 hyperplastic polyps, 15 tubulovillous adenomas, and 17 tubular adenomas). Of the 32 adenomas, 9 were classified as having mild, 16 moderate, and the remaining 7 severe dysplasia.

The hyperplastic polyps were uniformly negative for TLP antigen expression (Table 1 and Figure 2B). In contrast, 31 of 32 (96.9%) adenomas reacted with anti-TLP serum (Table 1). In all cases, the base of adenomas did not show any positive staining. One example of normal mucosa at the adenoma implant and one adenoma with low degree of dysplasia are shown in Figure 2, C and D.

Of interest, heterogeneous TLP expression related to the degree of dysplasia was observed. In fact, the percentage of positive cells ranged from 25 to 40% (mean, 33%) in adenomas with mild dysplasia, from 20 to 67% (mean, 50%) in adenomas with moderate dysplasia, and

from 78 to 92% (mean, 85.1%) in adenomas with severe dysplasia (Table 1). Moreover, different degrees of dysplasia were found to be associated with different patterns and intensity of immunohistochemical staining. As described in Materials and Methods, two different staining patterns were observed: supranuclear and diffuse (Figure 3). A supranuclear staining pattern was observed in 77.8% and 37.5% of adenomas with mild and moderate degree of dysplasia, respectively.

In contrast, 22.2% of cases of adenomas with mild dysplasia had a diffuse staining pattern, compared to 62.5% of adenomas with moderate degree of dysplasia (Table 1). All adenomas with severe dysplasia revealed a characteristic diffuse staining pattern and a strong staining intensity (+++).

No differences in the percentage of positive cases and/or positive cells were observed between tubular and tubulovillous adenomas (data not shown).

Figure 3. Immunohistochemical evaluation of TLP expression in three different cases of adenomas showing mild (A), moderate (B), and severe (C) epithelial dysplasia. All panels were hematoxylin-counterstained. Magnifications, ×630.

Colorectal Carcinomas

Tissue biopsies from 43 adenocarcinomas were analyzed for the presence of TLP antigen. A high percentage of cases (88.4%) showed a positive and homogeneous TLP expression (Table 1). Only few cases were found to be TLP-negative (11.6%). Of interest, when a stratification based on the degree of differentiation was performed, all negative cases were identified as poorly differentiated adenocarcinomas. Moreover, an analysis of the corresponding histologically normal colonic mucosa adjacent to the adenocarcinoma was performed in 21 cases (Table 1). Of these, 13 (61.9%) resulted TLP-negative, and

Figure 4. Immunohistochemical evaluation of TLP expression in normal colonic mucosa (A) from a patient with colorectal carcinoma and in two different cases of colorectal carcinoma (B and C). All panels were hematoxylin-counterstained. Magnifications, ×400.

only weak immunohistochemical staining for the antigen (staining intensity +) was obtained in the remaining 8 cases. In all 8 cases, TLP expression was found especially in the crypts. In addition, only a supranuclear staining pattern, comparable to that observed in the adenomas with mild dysplasia, was seen. Figure 4 reports an example of immunohistochemical analysis of a normal mucosa from a cancer patient (Figure 4A) and on two different tumor tissues (Figure 4, B and C). As shown, a high (+++) and homogeneous staining intensity was

observed in these as well as all the other positive adenocarcinomas.

Discussion

In the past two decades a wide variety of tumor-associated antigens has been characterized. Some of these molecules are not only expressed at the tissue level, but also shed into the biological fluids.^{1,7-13} Among those, TLP has been suggested to be a potential new tumor antigen. In fact, a recent report by Garaci et al¹⁷ demonstrated that the TLP antigen was present in sera of patients diagnosed with a variety of malignant diseases, including lung and colorectal carcinoma. Moreover, in that report the presence of this antigen in the serum correlated with early stages of malignant disease. Generally, the expression of tumor markers is related to the tumor burden; indeed, elevated levels of tumor markers are detectable mainly in sera of patients with advanced stages of cancer. Therefore, the finding that TLP is present in early stages of human cancer may suggest a possible association between TLP expression and the early event of cancer transformation. These findings focused our attention on the evaluation of TLP pattern expression during the process of cellular cancer transformation in one of the most studied models: the polyp-adenoma-carcinoma sequence in the colon and rectum. As reported in Table 1, none of the colonic mucosa from healthy donors scored positive for the presence of TLP antigen; furthermore, when inflammatory polyps were evaluated, similar results were obtained. This observation is of great interest, because most known tumor-associated antigens are also expressed by normal tissues.^{1,2,24} The results shown in Table 1 demonstrated also that the presence of TLP expression correlated with the degree of dysplasia. In fact, not only an increasing expression of the antigen, but a distinctive change in the subcellular localization of the antigen staining pattern (supranuclear versus diffuse) (Figure 3) as well as an increase in the relative intensity paralleled the degree of dysplasia (Table 1). Other tumor antigens, such as CEA and TAG-72, were found to be expressed in premalignant colorectal lesions and to be associated with the degree of dysplasia.^{1,2,4,5} The results obtained in this study are suggestive of a distribution of TLP antigen similar to that of TAG-72 antigen. In fact, TAG-72 is rarely expressed in normal adult tissues and its presence in premalignant colorectal lesions correlates with the degree of dysplasia.^{4,5} An increasing expression of distinctive antigens directly related to the degree of dysplasia may not only be of clinical relevance, but may also provide some insights in the process of colorectal cancer transformation.

Evaluation of the malignant colorectal tissues demonstrated that TLP expression is also related to the degree of cancer differentiation. In fact, as shown in Table 1, a tendency toward a greater proportion of TLP-positive tumors was observed among the highly differentiated tumors as compared to the less differentiated carcinomas. The loss of TLP immunoreactivity in poorly differentiated tumors may be explained by considering that an-

tigen expression includes highly complex parameters, such as synthesis, processing, membrane insertion, and active extrusion into the extracellular spaces, which may be lost during the dedifferentiation process. This finding is in agreement with those obtained for the majority of tumor-associated antigens studied.^{1,2,25} The high percentage of positive cells found in colorectal cancer suggests that TLP may be considered a new potential target for either diagnostic or therapeutic approaches to human cancer.

Furthermore, as has been reported for other tumor markers, some of the histologically normal colonic mucosa adjacent to the tumor was TLP-positive. Several hypotheses may be drawn; among them, we may consider the expression of this antigen as an epiphenomenon of cancer transformation, meaning that TLP expression can represent an early marker in the process of cancer cell transformation, as a product of genetic instability or initial genetic mutations.

In conclusion, our findings suggest that the TLP antigen could be regarded as a marker of epithelial atypia of the colon that occurs in the early phases of cancer transformation.

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References

1. Sikorska H, Shuster J, Gold P: Clinical applications of carcinoembryonic antigen. *Cancer Detect Prev* 1988, 12:321-355
2. Guadagni F, Roselli M, Cosimelli M, Spila A, Cavaliere F, D'Alessandro R, Arcuri R, Casale V, Fracasso P, Vecchione A, Casciani CU, Greiner JW, Schlom J: Quantitative analysis of CEA expression in colorectal adenocarcinoma and serum: lack of correlation. *Int J Cancer* 1997, 72:949-954
3. Stramignoni D, Bowen R, Atkinson B, Schlom J: Differential reactivity of monoclonal antibodies with human colon adenocarcinomas and adenomas. *Int J Cancer* 1983, 31:543-552
4. Wolf BC, D'Emilia JC, Salem RR, DeCoste D, Sears HF, Gottlieb LS, Steele GD Jr: Detection of the tumor-associated antigen glycoprotein antigen (TAG-72) in premalignant lesions of the colon. *J Natl Cancer Inst* 1989, 81:1913-1917
5. Guadagni F, Roselli M, Cosimelli M, Spila A, Cavaliere F, Tedesco M, Arcuri R, Abbolito MR, Casale V, Pericoli MN, Vecchione A, Casciani CU, Greiner JW, Schlom J: Correlation between tumor-associated glycoprotein 72 mucin levels in tumor and serum of colorectal cancer patients as measured by the quantitative CA 72-4 immunoassay. *Cancer Res* 1996, 56:5293-5298
6. Fischbach W, Mossner J: Tissue concentration of CEA and CA 19-9 in the carcinogenesis of colorectal carcinoma exemplified by the adenoma-carcinoma sequence. *Res Exp Med* 1988, 188:101-114
7. Mach JP, Jaeger P, Bertholet MM: Detection of recurrence of large-bowel carcinoma by radioimmunoassay of circulating carcinoembryonic antigen (CEA). *Lancet* 1974, 7:535-540
8. Martin EW Jr, Cooperman M, Carly LC, Minton JP: Sixty second-look procedures indicate primarily by rise in serial carcinoembryonic antigen. *J Surg Res* 1980, 28:389-394
9. Wood CB, Ratcliffe JG, Burt RW, Malcolm AJH, Blumgast LH: The clinical significance of the pattern of elevated serum carcinoembryonic antigen (CEA) levels in recurrent colorectal cancer. *Br J Surg* 1980, 67:47-48

10. Guadagni F, Roselli M, Amato T, Cosimelli M, Mannella E, Perri P, Abbolito MR, Cavaliere R, Colcher D, Greiner JW, Schlom J: Tumor-associated glycoprotein-72 serum levels complement carcinoembryonic antigen levels in monitoring patients with gastrointestinal carcinoma: A longitudinal study. *Cancer* 1991, 68:2443-2450
11. Guadagni F, Roselli M, Cosimelli M, Mannella E, Tedesco M, Cavaliere F, Grassi A, Abbolito MR, Greiner JW, Schlom J: TAG-72 (CA 72-4 assay) as a complementary serum tumor antigen to carcinoembryonic antigen in monitoring patients with colorectal cancer. *Cancer* 1993, 72:2098-2106
12. Herlyn M, Sears HF, Steplewski Z, Koprowski H: Monoclonal antibody detection of a circulating tumor-associated antigen. I. Presence of antigen in sera of patients with colorectal, gastric, and pancreatic carcinoma. *J Clin Immunol* 1982, 2:135-140
13. Sears HF, Herlyn M, Del Villano B, Steplewski Z, Koprowski H: Monoclonal antibody detection of a circulating tumor associated antigen. II. A longitudinal evaluation of patients with colorectal cancer. *J Clin Immunol* 1982, 2:141-149
14. Tarro G, Pederzini A, Flaminio G, Maturo S: Human tumor antigens inducing in vivo delayed hypersensitivity and in vitro mitogenic activity. *Oncology* 1983, 40:248-254
15. Tarro G, Marshak DR, Perna A, Esposito C: Antigenic regions of tumor liberated protein complexes and antibodies against the same. *Biomed Pharmacother* 1993, 47:237-240
16. Tarro G, Esposito C, Perna A, Claudio PP, Giordano A: Immunohistochemical characterization of tumor liberated particles (TLP) expression pattern in lung cancer. *Anticancer Res* 1998, 18:2365-2370
17. Garaci E, Sinibaldi P, Rasi G: A new tumour associated antigen of non-small cell lung cancer: Tumour liberated proteins (TLP): a possible new tumor marker. *Anticancer Res* 1996, 16:2253-2256
18. Morson BC, Jass JR: Precancerous lesions of the gastrointestinal tract: a histological classification. London, Bailliere, Tindall & Cox, 1985
19. Morson BC, Sobin LH: Histological typing of intestinal tumours. International Histological Classification of Tumours, no. 15. Geneva, World Health Organization, 1976
20. Konishi F, Morson BC: Pathology of colorectal adenomas: a colonoscopic survey. *J Clin Pathol* 1982, 35:830-841
21. Kuroki M, Fernsten PD, Wunderlich D, Colcher D, Simpson JF, Poole DJ, Schlom J: Serologic mapping of the TAG-72 tumor-associated antigen using 19 distinct monoclonal antibodies. *Cancer Res* 1990, 50:4872-4879
22. Muraro R, Wunderlich D, Thor A, Lundy J, Noguchi P, Cunningham R, Schlom J: Definition by monoclonal antibodies of a repertoire of epitopes on carcinoembryonic antigen differentially expressed in human colon carcinomas versus normal adult tissues. *Cancer Res* 1985, 45:5769-5780
23. Hsu SM, Raine L, Fanger H: Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody (PA) procedures. *J Histochem Cytochem* 1981, 29:577-580
24. Wolf BC, Salem RR, Sears HF, Horst DA, Lavin PT, Herlyn M, Itzkovitz SH, Schlom J, Steele G Jr: Expression of colorectal carcinoma (CRC)-associated antigens in the normal colon. *Am J Pathol* 1989, 135:111-119
25. Lundy J, Thor A, Maenza R, Schlom J, Forouhar F, Testa M, Kufe D: Monoclonal antibody DF3 correlates with tumor differentiation and hormone receptor status in breast cancer patients. *Breast Cancer Res Treat* 1985, 5:269-276