

Distinct expression pattern of the full set of secreted phospholipases A₂ in human colorectal adenocarcinomas: sPLA₂-III as a biomarker candidate

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Recent studies suggest that secreted phospholipases A₂ (sPLA₂s) represent attractive potential tumour biomarkers and therapeutic targets for various cancers. As a first step to address this issue in human colorectal cancer, we examined the expression of the full set of sPLA₂s in sporadic adenocarcinomas and normal matched mucosa from 21 patients by quantitative PCR and immunohistochemistry. In normal colon, PLA2G2A and PLA2G12A were expressed at high levels, PLA2G2D, PLA2G5, PLA2G10 and PLA2G12B at moderate levels, and PLA2G1B, PLA2G2F and PLA2G3 at low levels. In adenocarcinomas from left and right colon, the expression of PLA2G3 was increased by up to 40-fold, while that of PLA2G2D and PLA2G5 was decreased by up to 23- and 14-fold. The variations of expression for sPLA₂-IID, sPLA₂-III and sPLA₂-V were confirmed at the protein level. The expression pattern of these sPLA₂s appeared to be linked respectively to the overexpression of *interleukin-8*, *defensin α6*, *survivin* and *matrilysin*, and downregulation of *SFRP-1* and *RLPA-1*, all these genes being associated to colon cancer. This original sPLA₂ profile observed in adenocarcinomas highlights the potential role of certain sPLA₂s in colon cancer and suggests that sPLA₂-III might be a good candidate as a novel biomarker for both left and right colon cancers.

British Journal of Cancer (2008) **98**, 587–595. doi:10.1038/sj.bjc.6604184 www.bjcancer.com

Published online 22 January 2008

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Keywords: colon cancer; secreted phospholipase A₂; gene expression; qPCR; immunohistochemistry; biomarker

Sporadic colorectal cancer is a major human health concern in industrialised countries and is the third leading cause of mortality by cancer. The accumulation of genetic alterations in several key genes (*APC*, *K-ras*, *DCC* and *p53*) has been associated with tumour development (Fearon and Vogelstein, 1990). In particular, the activation of the Wnt/ β -catenin/Tcf-4 pathway is known to play a key role in colon tumorigenesis, leading to overexpression of a large number of target genes, including *c-myc*, *cyclin D1*, *MMP-7*, *ITF-2*, *IL-8*, *gastrin*, *uPAR*, *cryptdin/defensin* and *EPR-1* genes (Kolligs *et al*, 2002; Levy *et al*, 2002). Moreover, the participation of tumour modifier genes may also contribute to pathogenesis, providing a basis to individual genetic predispositions, in addition to environmental and diet influences (Wiesner *et al*, 2003; Yan *et al*, 2004; Rakoff-Nahoum and Medzhitov, 2007). A comprehensive characterisation of such tumour modifier genes must take into account the stage of cancer and the location of the tumour within the large intestine, that is, 'right' or 'left' colon. This information may lead to a better understanding of the molecular events involved in colorectal cancer, and may provide new therapeutic targets to improve patient

treatment outcomes (Williams *et al*, 2003; Barrier *et al*, 2005). It is also of interest to investigate the expression of such genes to identify new biomarkers of human colorectal cancers for early diagnosis and prognosis (Williams *et al*, 2003; Barrier *et al*, 2005).

In humans, 10 secreted phospholipases A₂ (sPLA₂s) have been described in the last decade, and the analysis of their functions in physiological and pathophysiological conditions is under intense investigation. Human sPLA₂s have been classified into groups IB, IIA, IID, IIE, IIF, III, V, X, XIIA and XIIB according to their structural properties (Laye and Gill, 2003; Rouault *et al*, 2003; Murakami *et al*, 2005; Cummings, 2007). Several of these proteins have recently been proposed either as biomarkers of pathologies (Smith *et al*, 2003; Mallat *et al*, 2005; Wootton *et al*, 2007) or as therapeutic targets (Laye and Gill, 2003; Cummings, 2007; Henderson *et al*, 2007). These enzymes have the capacity to generate biologically active lipid mediators such as lysophosphatidic acid (LPA) and arachidonic acid (AA), which can be further converted into prostaglandin E₂ (PGE₂). These lipid mediators are known to be involved in cell proliferation, cell migration, angiogenesis, and are likely to play a role in the initiation and/or progression of colorectal cancer (Morioka *et al*, 2000; Krause and DuBois, 2001; Laye and Gill, 2003; Mills and Moolenaar, 2003; Murakami *et al*, 2005). AA can also participate in apoptosis by activating sphingomyelinases and ceramide production (Dong *et al*, 2005; Ilsley *et al*, 2005).

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Received 2 October 2007; revised 12 December 2007; accepted 12 December 2007; published online 22 January 2008

Secreted phospholipases A₂ have been recently proposed as targets for anticancer drugs (Laye and Gill, 2003; Cummings, 2007), and there is increasing evidence for their involvement in various human cancers. Indeed, the expression levels of sPLA₂-V and sPLA₂-X are modified in human lung cancer (Masuda *et al*, 2005b), and those of sPLA₂-V and sPLA₂-XIIA in human ovarian cancer (Gorovetz *et al*, 2006). In particular, the overexpression of human sPLA₂-IIA in gastric adenocarcinoma was proposed to be related to prolonged survival and less frequent metastasis (Leung *et al*, 2002; Aggarwal *et al*, 2006). By contrast, sPLA₂-IIA overexpression has been associated to oncogenic effects in prostate cancer (Sved *et al*, 2004) and is related to poor prognosis (Graff *et al*, 2001). The polymorphism of the sPLA₂-IIA gene, *PLA2G2A*, is also associated to some phenotypic features of patients with familial adenomatous polyposis (Yanaru-Fujisawa *et al*, 2007). More recently, human sPLA₂-XIIB was shown to be upregulated in 50% of patients with hepatitis C virus-associated hepatocellular carcinoma (Smith *et al*, 2003). Finally, a protective role for sPLA₂-IIA against *Apc*^{Min}-induced intestinal tumours has been established in mice (MacPhee *et al*, 1995; Cormier *et al*, 1997). However, data on sPLA₂ and human cancers are still sparse, and often rely on only one or a few sPLA₂s.

In this study, we have analysed the expression pattern of the full set of sPLA₂s in tumour vs normal matched mucosa from patients with adenocarcinomas located in the left and right colon. Variation in the expression of a number of other genes associated with colon tumorigenesis and/or inflammation was also examined to establish possible gene coregulations. Our data provide the first comprehensive analysis of the expression pattern of all sPLA₂s in normal human colon mucosa as well as adenocarcinomas. These data further support the fact that several sPLA₂s may contribute to the initiation, progression or modulation of colon tumorigenesis, and may provide new potential tumour markers for this disease.

MATERIALS AND METHODS

Human tissues and RNA isolation

Fourteen colon adenocarcinoma specimens from the left colon (descending part of the colon) and seven colon adenocarcinoma specimens from the right colon (ascending part of the colon) were obtained from surgical resections according to the French and American institutional guidelines. No specimen was from rectum. The patients did not receive chemotherapy or radiotherapy prior to surgery. The specimens were from 42- to 85-year-old patients (median age: 71 years), 14 men and 7 women. Samples were well (3 out of 21), moderately (14 out of 21) or poorly (4 out of 21) differentiated sporadic adenocarcinomas, with pTNM classification ranging pT2–4 N0–2 M0–1. Neither adenoma nor Duke'D adenocarcinoma was examined in this study. All samples had an expression of hMLH1 and hMSH2 unchanged as evaluated by immunohistochemistry and quantitative PCR (qPCR) (data not shown). Tissues from the non-necrotic part of the tumour and from distant normal mucosa were snap frozen in liquid nitrogen and stored at –80°C. Each tissue sample (20–100 mg) was mixed with 700 μl of lysis buffer plus β-mercaptoethanol (nucleospin RNA II kit; Macherey-Nagel, Hoerdt, France) in a green-cap tube containing Lysing Matrix D (Q-BIOgene, Illkrich, France), and tissue disruption was achieved with the fast prep instrument (FP220A; Q-BIOgene). Total RNA was then isolated using the nucleospin RNA II kit, including DNase treatment. RNA concentration was determined by OD₂₆₀ and RNA quality was evaluated by analysis on an Agilent Bioanalyzer (Agilent Technologies, Les Ulis, France).

Quantitative PCR

First-strand cDNA was synthesised from 5 μg of total RNA using 100 U of MMLV reverse transcriptase (#M170A; Promega,

Charbonnières-les bains, France) in a final volume of 50 μl with 500 ng of random primers (#C118A; Promega). Quantitative PCR was carried out in 96-well ABgene plates using the GENEAMP 5700 sequence Detection System apparatus (Applied Biosystems, Courtaboeuf, France) with the qPCR Master Mix Plus for SYBR[®] Green I (Eurogentec, Angers, France). All reactions were performed in a total volume of 16 μl and contained 50 ng of reverse transcribed RNA (based on the initial RNA concentration) and 250 nM of each primer set. The primer sets were designed using the Primer Express program from Applied Biosystems for the following human genes: *PLA2G2A* (NM_000300), sPLA₂-IID gene (*PLA2G2D*) (NM_012400), sPLA₂-IIE gene (*PLA2G2E*) (NM_0145891), sPLA₂-IIF gene (*PLA2G2F*) (NM_022819), sPLA₂-III gene (*PLA2G3*) (NM_015715), sPLA₂-V gene (*PLA2G5*) (NM_000929), sPLA₂-X gene (*PLA2G10*) (NM_003561), sPLA₂-XIIA gene (*PLA2G12A*) (BC_017218), sPLA₂-XIIB gene (*PLA2G12B*) (NM_032562), iPLA₂-VIB gene (*PLA2G6*) (NM_003560), *ptgs1* (BC_029840), COX-2 gene (*ptgs2*) (NM_000963), *ptgs1* (NM_004878), *ptgs2* (NM_198797), *IL-1α* (NM_000575), *IL-6* (NM_000600), *IL-10* (NM_57627), *PPARγ* (NM_138712), *PPARδ* (NM_006238), *u-PA* (NM_002658), *u-PAR* (NM_002659), *IL-8* (NM_000584), *EPR-1* (NM_002219), *MMP-7* (NM_002423), *SFRP-1* (NM_003012), *PLA2R1* (NM_007366), *TNF-α* (NM_000594), *MMP-9* (NM_004994), *MSH2* (NM_000251), *MLH1* (NM_000249). Most primer sets were designed to span an intron in order to avoid amplification from potential traces of genomic DNA in the total RNA preparations. Only the primer sets for *MLH1*, *uPAR*, *EPR-1*, *MMP-7* and *bcl-2* genes were not spanning an intron. For primer sets spanning an intron, we checked that no amplification signal was obtained using human genomic DNA as template in the qPCR (data not shown). The sequences of the designed primer sets are available on request. We used Qiagen commercial primer sets for cPLA₂-IVA gene (*PLA2G4A*) (ref. QT00085813) and sPLA₂-IB gene (*PLA2G1B*) (ref. QT00000637). We used published primer sets for *RLPA-1* and *RLPA-2* genes (Shida *et al*, 2004) and *HD-6* gene (Andreu *et al*, 2005). The efficiency and specificity of each primer sets were validated using either serial dilutions of cloned human sPLA₂ cDNAs or mixed human tissue cDNA for the other genes. Moreover, when enough total RNA was collected, negative controls without added reverse transcriptase were performed. Thermal cycling was performed at 95°C for 10 min, followed by 40 cycles comprising each a denaturation step at 95°C for 15 s, and an annealing/extension step at 60°C for 1 min. Amplification of the appropriate product was verified by analysing the dissociation curve that was obtained after PCR with the following steps: 15 s at 95°C, 20 s at 60°C, and then a slow ramp of 20 min from 60 to 95°C. The abundance of the mRNA target was calculated relative to the expression of the reference gene *TOP1* and is expressed as 2^{–ΔC_t}, where ΔC_t = C_t (gene of interest) – C_t (*TOP1*). The choice of *TOP1* as a reference gene was determined using a Human GeNorm kit (PrimerDesign Ltd, Southampton, UK), allowing the determination of the best reference gene among 12 widely used reference genes. The data were also validated using *GAPDH* as a reference gene (data not shown). When the relative level of expression was plotted for normal mucosa (n) (Figure 1), we used the formula 2^{20–ΔC_t(n)}, with ΔC_t = C_t(n)gene – C_t(n)*TOP1*. The C_t(n) values for *TOP1* were typically around 20. When the expression of each gene in normal mucosa vs tumours (Figures 2–4) was plotted, the comparative C_t method (detailed in the ABI Prism 7700 Sequence Detection System User Bulletin no. 2) was used to determine the relative quantities of mRNA, and we changed the sign in order to get the lowest level of expression at the bottom and the highest level of expression at the top of the y axis, that is, –ΔC_t = –(C_t gene of interest – C_t *TOP1*). When the expression of each gene was compared between the tumour (t) and the matched normal mucosa (n), the decrease or increase factor φ in the tumour vs the normal mucosa (Figure 5A and B) was calculated with the formula

$$\phi = 2^{I\Delta C_t(n) - \Delta C_t(t)I - 1}, \quad \text{with} \quad \Delta C_t(n) = C_t(n)\text{gene} - C_t(n)\text{TOP1}, \\ \Delta C_t(t) = C_t(t)\text{gene} - C_t(t)\text{TOP1}, \quad \text{and} \quad I \text{ the absolute value of the term } \Delta C_t(n) - \Delta C_t(t).$$

Immunohistochemistry

All experiments were performed using paraffin-embedded tissues from the same patients as those used for the qPCR experiments.

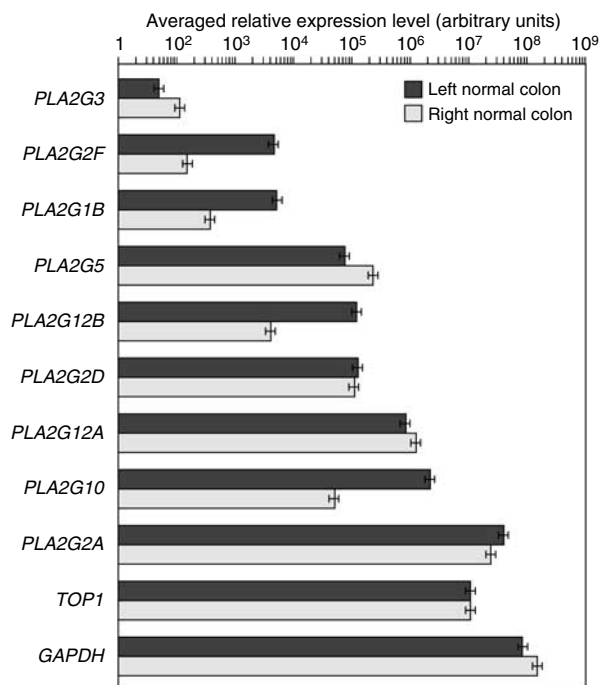


Figure 1 Expression level of sPLA₂ genes in human colon normal mucosa. The relative averaged expression level of all sPLA₂ genes and the two reference genes used in this study (*TOP1* and *GAPDH*) is shown for the 14 samples from left colon and the 7 samples from right colon. Arbitrary units are used (see Materials and Methods).

The expression of sPLA₂-IID, sPLA₂-III, sPLA₂-V and COX-2 proteins was analysed in tumours and normal matched tissues from three to four patients. Consecutive 4- μ m tissue sections were deparaffinised in xylene and rehydrated in graded alcohol dilutions. Immunolabelling was performed using avidin-biotin-peroxidase technique (Vectastain ABC kit; Vector, Burlingame, CA, USA). Before immunostaining, endogenous peroxidase activity was inhibited with 0.1% hydrogen peroxide in methanol for 30 min. Colour development was achieved with 3-amino-9-ethylcarbazole, and sections were finally counterstained with haematoxylin. Specific rabbit polyclonal antibodies against recombinant human sPLA₂-IID, sPLA₂-III and sPLA₂-V were produced as described (Haas *et al*, 2005), and used at working dilutions of 1/300, 1/250 and 1/100, respectively. All anti-sPLA₂ antibodies were tested for specificity towards the various sPLA₂s and were shown to be highly specific for each human sPLA₂ (Haas *et al*, 2005). Negative controls were performed by omission of the primary antibody. To further check for the specificity of labelling with sPLA₂-IID-, sPLA₂-III- and sPLA₂-V-specific antibodies, competition experiments were performed in which the antibody was preincubated with the corresponding purified recombinant sPLA₂ used for immunisation, prior to covering the tissue slides. The competition was performed by preincubating the relevant antibody solution with 200 nM of human recombinant sPLA₂-III or 100 nM of human recombinant sPLA₂-IID and sPLA₂-V for 1 h at room temperature. Purified recombinant proteins were obtained as described (Rouault *et al*, 2007). Human COX-2 antibodies were from Cayman (Montigny-le Bretonneux, France) (ref. 160112, working dilution 1/500), human MLH1 antibodies were from BD Pharmingen (Le Pont-de-Claix, France) (clone G168-178, working dilution 1/100), and human MSH2 antibodies were from Calbiochem (Darmstadt, Germany) (clone FE-11, working dilution 1/125).

Statistics

We determined that the sample data from left and right colons followed a Gaussian distribution using the D'agostino-Pearson normality test. Normal mucosa and tumour paired data for each gene were analysed using the Bonferroni test, and statistical significances were represented by * $P < 0.05$, ** $P < 0.01$ and

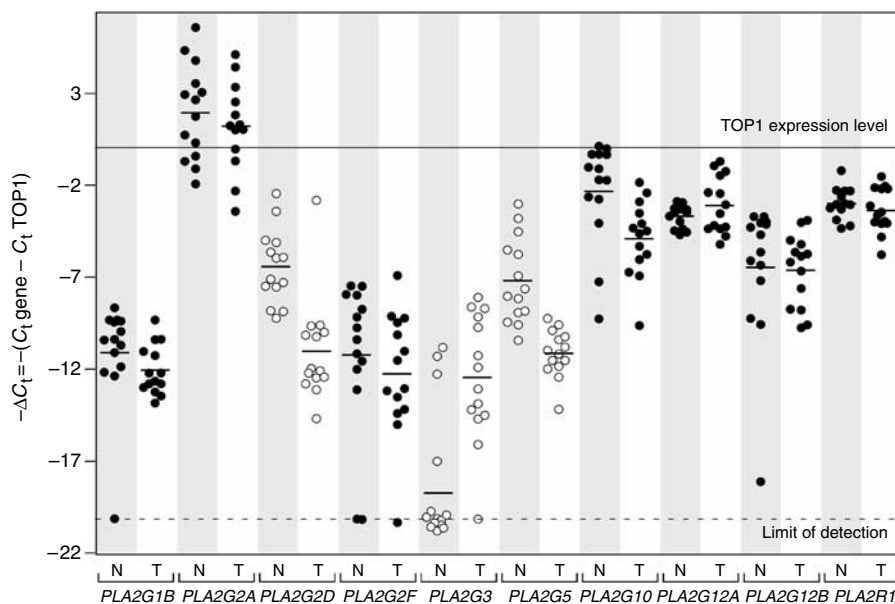


Figure 2 Expression level of sPLA₂ genes in human adenocarcinomas and normal matched mucosa. The expression level of sPLA₂ genes and of the M-type sPLA₂ receptor (*PLA2R1*) was measured in adenocarcinomas and normal matched mucosa for the 14 left-sided samples. The data were obtained after normalisation with *TOP1* used as reference gene and using the formula $-\Delta C_t = C_t \text{ gene} - C_t \text{ TOP1}$. N, normal tissues; T, tumour tissues.

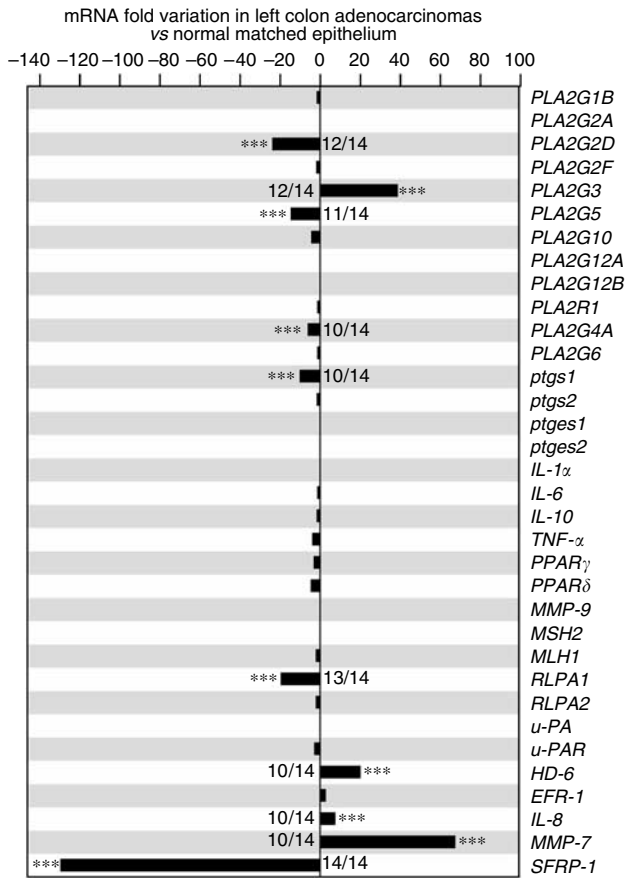


Figure 3 Expression level of sPLA₂, tumour-related and inflammation-related genes in human left colon adenocarcinomas. After normalisation with the *TOP1* reference gene, the expression level for each gene in the tumour was compared to that in the normal matched mucosa. The zero value indicates no variation, a positive value indicates an increased expression level in tumour vs normal tissue and a negative value indicates a decreased expression level in tumour vs normal tissue (see Materials and Methods). The incidence of variation in the different samples is indicated.

*** $P < 0.001$. We looked for correlation between all genes examined in this study by using the Mev3.1 software and hierarchical clustering to analyse different linkages to cluster genes and samples. Euclidean distance was used to calculate the distance between two genes.

RESULTS

Expression levels of sPLA₂ genes in normal human colon mucosa

We first looked at the mRNA levels for the different sPLA₂ genes in normal mucosa and compared their relative expression levels. Our data show dramatic differences in the relative expression of sPLA₂. The lowest gene expression level was found for *PLA2G3*, and the highest gene expression level was found for *PLA2G2A* (Figure 1). The level of *PLA2G2A* expression was particularly high. It is above that of *TOP1* and almost reaches that of *GAPDH*, which are both used as reference genes and are known to be highly expressed in many tissues, including colon. By contrast, the expression of *PLA2G3* was generally very low, in fact below the limit of detection in many samples (Figure 1). The expression levels of *PLA2G2F* and *PLA2G1B* were also low, while that of other sPLA₂ genes were moderate. The expression of *PLA2G2E* was not detectable (data not

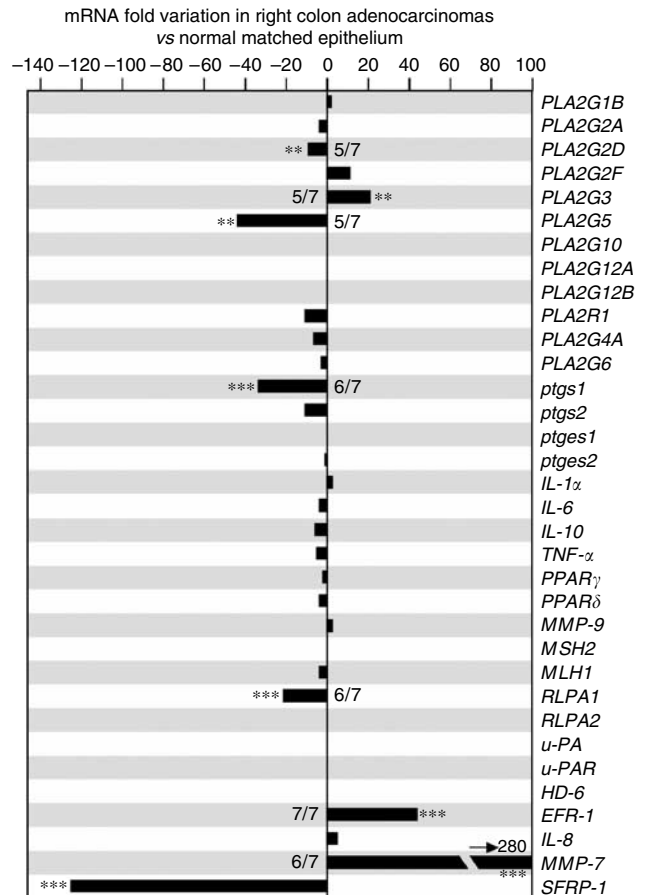


Figure 4 Expression level of sPLA₂, tumour-related and inflammation-related genes in human right colon adenocarcinomas. Same legend as Figure 3.

shown). We also compared the expression levels in normal mucosa of left and right colon. A significant difference in gene expression levels was seen for *PLA2G2F*, *PLA2G1B*, *PLA2G12B* and *PLA2G10* (respectively 30-, 13-, 24- and 40-fold lower expression in right-sided normal mucosa than in left-sided normal mucosa). No or minor differences were observed in gene expression for *PLA2G2A*, *PLA2G2D*, *PLA2G3*, *PLA2G5* and *PLA2G12A* between right- and left-sided normal mucosa (Figure 1).

Expression levels of sPLA₂ genes in left and right colon adenocarcinomas vs normal human colon mucosa

We next compared sPLA₂ gene expression in adenocarcinomas vs normal mucosa. The raw data for samples from the left colon are shown in Figure 2. The fold-increase or fold-decrease in the expression of each gene in adenocarcinomas vs normal mucosa is represented in Figure 3. Interestingly, we observed a 40-fold increase in *PLA2G3* expression in the adenocarcinomas. In contrast, we found 23- and 14-fold decreases for *PLA2G2D* and *PLA2G5*, respectively. Similar data were obtained for samples from the right colon, with a 22-fold increased expression in tumour vs normal mucosa for *PLA2G3*, and 10- and 44-fold reduced expression for *PLA2G2D* and *PLA2G5*, respectively (Figure 4). No variation in expression between the tumours and normal mucosa was observed for *PLA2G1B*, *PLA2G2A*, *PLA2G2F*, *PLA2G10*, *PLA2G12A* and *PLA2G12B* and for the M-type sPLA₂ receptor (*PLA2R1*) (Figures 3 and 4). The expression of *PLA2G2E*

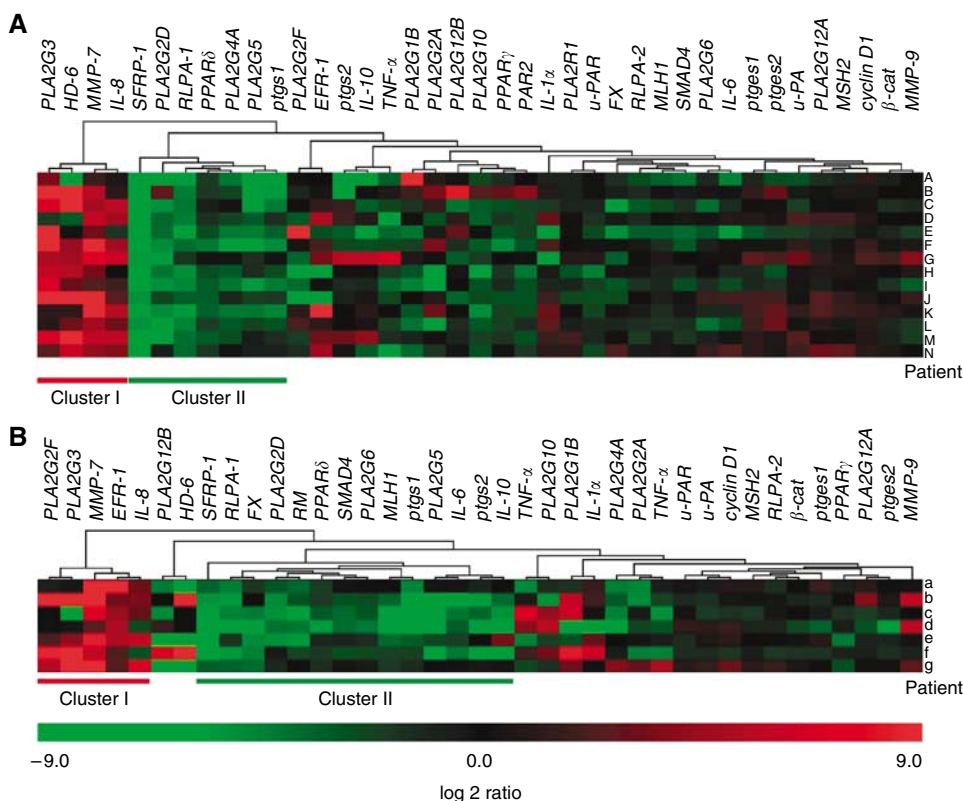


Figure 5 Hierarchical clustering of genes. Heatmaps comparing the log₂ ratio between the expression level in adenocarcinomas and normal epithelium for the 14 patients with left-sided adenocarcinomas (**A**) and for the 7 patients with right-sided adenocarcinomas (**B**) are shown. The distance corresponds to an Euclidean distance calculated using the Mev3.1 software.

was not detectable in any of the samples analysed (data not shown).

Expression levels of genes involved in inflammation and tumorigenesis in left and right colon adenocarcinomas vs normal human colon mucosa

Since sPLA₂s may exert a coordinate action with other genes in tumorigenesis and associated inflammation, we also examined the expression of a panel of genes known to be involved in inflammation and colorectal tumorigenesis (Figures 3 and 4). In left colon samples, we did not observe any differences in expression levels between adenocarcinomas and normal mucosa for most of the inflammation-related genes examined in this study, that is, *ptgs2*, *ptgs1*, *ptges2*, *TNF-α*, *IL-1α*, *IL-6*, *IL-10*, *PPARγ*, *PPARδ*, *MMP-9* and *PLA2G6*. Only a slight decrease in the expression of *ptgs1* and *PLA2G4A* was observed. With the exception of *ptgs2* and *IL-1α*, we found that the inflammation-related genes were already expressed at high levels in the normal mucosa.

We also looked at the expression of urokinase plasminogen activator (*u-PA*) and its receptor (*u-PAR*) that play a role in cell adhesion and cell migration, and are involved in late stages of tumour development, contributing to tumour cell invasion and metastatic spread (Terada *et al*, 2005). No changes were observed in *u-PA* and *u-PAR* gene expression levels.

RLPA-1 and *RLPA-2* genes code for two distinct LPA receptors (Mills and Moolenaar, 2003). While the expression of *RLPA-2* did not change, that of *RLPA-1* was decreased by 20-fold in tumour vs normal matched mucosa.

IL-8, *EPR-1*, *HD-6* and *MMP-7* are target genes of the Wnt/ β -cat/tcf-4 pathway (Kolligs *et al*, 2002; Levy *et al*, 2002; Wheatley and

McNeish, 2005). Interestingly, a marked increase in expression was found for *IL-8*, *HD-6* and *MMP-7* expression in the tumours (8-, 22- and 70-fold, respectively). The level of *EPR-1* was not changed. *SFRP-1* codes for an antagonist of the Wnt pathway, which binds to the frizzled receptor and blocks frizzled–Wnt interaction (Suzuki *et al*, 2004). Our results show a dramatic 130-fold decrease in the expression of *SFRP-1* in the tumour.

Similar data were obtained for most genes in the right-sided samples (Figure 4). Indeed, no variation in expression was observed for most inflammatory-related genes, while a dramatic increase (280-fold) and a strong decrease (125-fold) were found for *MMP-7* and *SFRP-1*, respectively. However, in contrast to left-sided samples, we observed no variation in *HD-6* and *IL-8* expression levels and a strong increase (45-fold) in *EPR-1* expression.

Incidence and coregulation of studied genes

We first looked at the incidence of the above variations, that is, the number of samples showing an increase or decrease in the expression level of a particular gene within the 21 patients used in this study (Figures 3–5). The increase in *PLA2G3* expression showed a high incidence (12 out of 14 for left-sided samples and 5 out of 7 for right-sided samples), which was similar to that of *MMP-7* gene overexpression (10 out of 14 for left-sided samples and 6 out of 7 for right-sided samples). The incidence for the overexpression of *HD-6* and *IL-8* was lower, since these two genes were not significantly upregulated in right-sided samples. The decrease in *PLA2G2D* and *PLA2G5* expression also showed a high incidence (12 out of 14 and 5 out of 7; and 11 out of 14 and 5 out of 7, respectively), which was similar to that of *RLPA-1* and *SFRP-1* gene expression (13 out of 14 and 6 out of 7; and 14 out of 14 and 7 out of 7, respectively). We did not observe any difference between

female and male patients used in our study for the variations of expression of *PLA2G3*, *PLA2G2D* and *PLA2G5* (data not shown).

Euclidean distance is commonly used to evaluate linkages in the expression of different genes. We used this method to point out linkages between the variations in expression level of the most relevant genes. In left-sided adenocarcinomas (Figure 5A), a first cluster (cluster I) of upregulated genes was observed containing *PLA2G3* and several genes related to colon cancer such as *HD-6*, *MMP-7* and *Il-8*. A second cluster (cluster II) of several downregulated genes, including *SFRP-1*, *PLA2G2D*, *RLPA-1*, *PLA2G5*, *PLA2G4A* and *ptgs1*, was observed. These two clusters appeared most likely to discriminate adenocarcinomas from normal tissues. Remarkably, there are 3 sPLA₂ genes out of 11 genes in these two clusters. The other genes did not show any linkage with colon tumorigenesis, since their expression levels were up- or downregulated or not changed among the different patients. When looking at right-sided samples (Figure 5B), similar patterns were observed even though the number of patients was lower.

Protein expression levels of sPLA₂-IID, sPLA₂-III and sPLA₂-V in colon adenocarcinomas and normal matched mucosa

To confirm that the variation of expression for *PLA2G2D*, *PLA2G3* and *PLA2G5* is also observed at the protein level, immunohistochemical analyses were performed on tissue sections from the same patients as those used for qPCR. For each sPLA₂ protein analysed, tissue sections from three or four patients showing a significant increase or decrease of sPLA₂ expression were selected and immunostained (Figure 6). The labelling for sPLA₂-IID and sPLA₂-V was markedly decreased in tumour cells compared to the normal matched epithelium, in accordance with the qPCR data. By contrast, the labelling for sPLA₂-III was absent or very low in the normal epithelium, with a significant increase in tumour tissue. The absence of labelling in competition experiments (data not shown) or when the primary antibody was omitted (Figure 6) demonstrated that the signals observed for the three sPLA₂ proteins were specific. In addition, immunohistochemical analysis of COX-2 showed a more intense protein expression in tumours than in normal mucosa in a subset of patients (data not shown).

DISCUSSION

Several lines of evidence have been accumulated during the past decade to support the role of sPLA₂s in cancer pathogenesis. Their role in human cancer, however, has not been clarified. As a first step towards addressing this issue, we have analysed the expression patterns of the full set of human sPLA₂s in colorectal cancer tissue samples and normal matched mucosa. Our results indicate that (i) several sPLA₂s, including sPLA₂-IIA, sPLA₂-X and sPLA₂-XIIA, are highly expressed in both normal and tumour colon tissues; (ii) the expression levels of sPLA₂-III, sPLA₂-IID and sPLA₂-V are dramatically altered in adenocarcinomas at both mRNA and protein levels; and (iii) their mRNA profiles are part of gene expression clusters with other genes associated with inflammation and cancer. Together, our data are further suggestive of a role of these sPLA₂s in colorectal cancer and open the possibility that sPLA₂s, in particular sPLA₂-III, may provide novel cancer biomarkers.

sPLA₂ gene expression levels in colon adenocarcinomas and normal human colon

Colon adenocarcinomas analysed in this study are likely cancers without high microsatellite instability. Indeed, we used immunohistochemical analysis as a reliable method for screening DNA

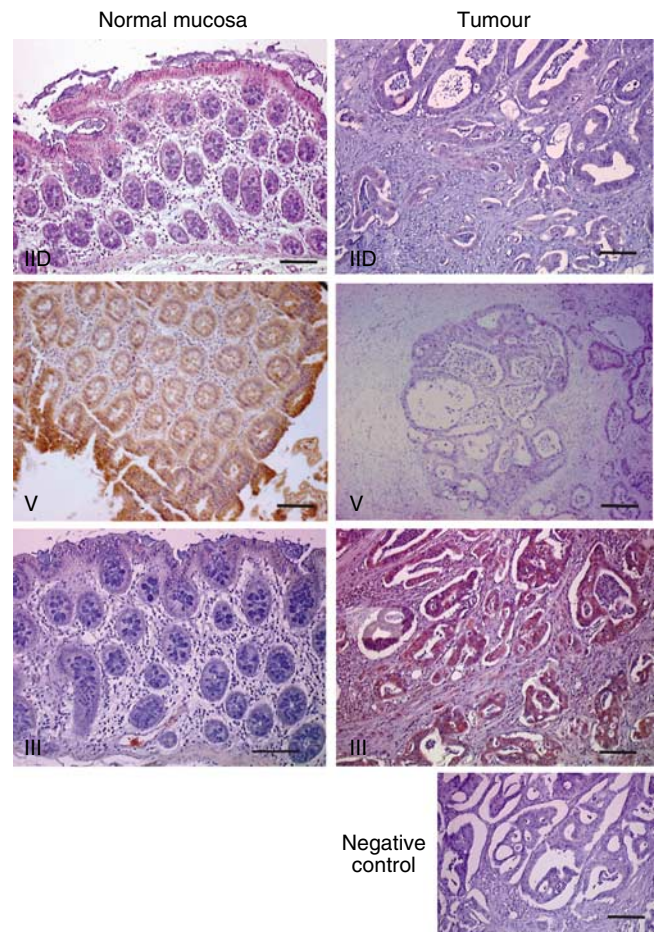


Figure 6 Secreted phospholipase A₂ immunolabelling in representative colon adenocarcinomas and normal matched mucosa. For sPLA₂-IID and sPLA₂-V expression, a strong staining in the surface epithelium of the normal mucosa was observed, contrasting with the weak or absent staining observed in tumour cells. For sPLA₂-III expression, a very faint staining was observed in normal epithelial cells, contrasting with the moderate to strong staining observed in tumour cells. For each sPLA₂, a representative immunolabelling from 1 patient out of 3–4 patients analysed is shown. As shown in the bottom picture, no staining is observed when the primary antibody is omitted. Scale bar is 100 μ m.

mismatch repair defects (Lindor *et al*, 2002), and found that the expression of hMLH1 and hMSH2 was unchanged. Furthermore, we analysed the normal mucosa and the tumour tissues from the right and left colon separately because of their distinct gene expression patterns (Glebov *et al*, 2003; Birkenkamp-Demtroder *et al*, 2005). We observed similar levels of expression for most sPLA₂s in left and right normal mucosa, with *PLA2G2A* having a high expression level, *PLA2G3* being not detectable in most samples and the other sPLA₂ genes showing intermediate levels of expression (Figure 1). Interestingly, we observed marked alterations in the expression levels of *PLA2G2D*, *PLA2G5* and *PLA2G3* in colon adenocarcinomas. In contrast, there was no difference in the expression levels of the other sPLA₂ genes (Figures 2–4). The absence of tumour-related alterations in the gene expression of *PLA2G2A* and *PLA2G10* in human sporadic colon cancer tissues is consistent with earlier studies (Dimberg *et al*, 1998; Osterstrom *et al*, 2002; Murakami *et al*, 2005). These data obtained in humans differ from those obtained in mice, which showed an increased expression of *PLA2G2A* and no variation in *PLA2G5* expression in tumours from azoxymethane-treated mice (Ilsley *et al*, 2005).

Expression level of genes involved in inflammation and tumorigenesis in colon adenocarcinomas and normal human colon

No significant changes between adenocarcinomas and normal colon were observed for most inflammation-related genes. It should be noted that many of these genes were expressed at high levels within the normal mucosa, in particular, *MMP-9*, *PLA2G6* and *ptgs2*. A recent study by Chen *et al* (2004) has also shown high mRNA expression levels of several inflammation-related genes in normal-appearing colon mucosa of patients with colon cancer. Therefore, our data would suggest a possible pre-existing inflammatory condition within the normal mucosa distant from the tumours, and likely explain the absence of increased expression in adenocarcinomas *vs* normal mucosa for several inflammation-related genes. The slight decrease in *ptgs1* (the COX-1 gene) appeared in agreement with that observed in colon adenocarcinomas of stage III (Duke's C) patients (Church *et al*, 2004). The COX-1 protein is considered to exert dual opposing effects in cancer, acting as either a tumour suppressor or a tumour initiator (Chulada *et al*, 2000). The slight decrease in *PLA2G4A* expression that we observed may be related to the dual opposing effects of cPLA₂-IVA in cancer, that is, proliferative effects via metabolism of AA into eicosanoids, such as PGE₂, and anti-proliferative effects via AA-dependent ceramide production leading to apoptosis (Ilsley *et al*, 2005). The absence of increase in *ptgs2* (the COX-2 gene) levels in our set of patients is in accordance with two recent qPCR studies also performed on patients with colon cancer (Church *et al*, 2004; Gustafsson *et al*, 2007), and may be related to the inflammatory status of the normal mucosa discussed above. The fact that we did not observe an increase of COX-2 at the mRNA level, while we detected an overexpression at the protein level by immunohistochemistry as previously reported (Wendum *et al*, 2003), is in line with the post-transcriptional regulation of COX-2 expression (Dixon *et al*, 2003). Therefore, mRNA and protein levels of COX-2 may not be closely linked, as recently proposed in colon cancer tissues (Gustafsson *et al*, 2007) and colon cancer cells (Dixon *et al*, 2003).

Downregulation of sPLA₂-IID and sPLA₂-V expression in human colon adenocarcinomas

A marked decrease in the expression level of *PLA2G2D* and *PLA2G5* was observed in both left- and right-sided adenocarcinomas (Figures 2–4). These variations were also observed at the protein level by immunohistochemical analyses. Indeed, sPLA₂-IID and sPLA₂-V were found to be present in epithelial cells of normal mucosa, and their expression was markedly reduced in tumours (Figure 6). The decreased expression of sPLA₂-IID and sPLA₂-V in tumours may suggest a protective role of these sPLA₂s, as it has been proposed for sPLA₂-IIA in mice (MacPhee *et al*, 1995; Cormier *et al*, 1997). A similar decrease in expression of sPLA₂-IID and sPLA₂-V was also described in gastric tissues with signet-ring cell carcinoma (Masuda *et al*, 2005a). Interestingly, the *PLA2G2A*, *PLA2G2C*, *PLA2G2D*, *PLA2G2E*, *PLA2G2F* and *PLA2G5* genes reside within the same region of human chromosome 1 at p35–36.1 (Valentin *et al*, 2000), a region frequently altered in colorectal cancer (Spirio *et al*, 1996). The decreased expression for *PLA2G2D* and *PLA2G5* appears to be linked (cluster II, Figure 5) to that observed for *PLA2G4A*, *ptgs1*, *SFRP-1* and *RLPA-1*, which have been proposed as tumour suppressor genes in colon cancer (Chulada *et al*, 2000; Shida *et al*, 2004; Suzuki *et al*, 2004; Dong *et al*, 2005).

Upregulation of PLA2G3 and Wnt target genes in human colon adenocarcinomas

A marked increase in sPLA₂-III expression level was observed in both left- and right-sided adenocarcinomas, a finding that was

confirmed by immunohistochemical analysis. No or very weak sPLA₂-III expression was observed in normal epithelial cells, but a robust expression was observed in tumours (Figure 6). Our findings are consistent with the recent observation that sPLA₂-III can trigger the proliferation of human colon cancer cells *in vitro* (Murakami *et al*, 2005). Parallel to the increase in *PLA2G3* gene expression level, we observed an increase in the expression level of four target genes of the Wnt/ β -cat/Tcf-4 pathway: *Il-8* (Levy *et al*, 2002), *HD-6* (also called defensin $\alpha 6$) (Kolligs *et al*, 2002), *MMP-7* (also called matrilysin) (Nelson *et al*, 2000) and *EPR-1* (also called survivin) (Kolligs *et al*, 2002), as well as a concomitant dramatic decrease in *SFRP-1* gene expression level. Therefore, our data are consistent with the activation of the Wnt/ β -cat/Tcf-4 pathway in the development of colon adenocarcinomas. It will be of interest to determine whether the expression of *PLA2G3* is related to the activation of the Wnt/ β -cat/Tcf-4 pathway, as already suggested for *PLA2G2A* in gastric cancer (Aggarwal *et al*, 2006).

Our data further support the role of LPA in the pathogenesis of colon cancer. LPA exerts its effects through at least three different receptors: RLPA-1, RLPA-2 and RLPA-3 (Mills and Moolenaar, 2003). It has been demonstrated that the Wnt/ β -cat/Tcf-4 pathway is involved in the proliferative effects of LPA through binding to RLPA-2 (Yang *et al*, 2005). We observed a decreased expression of *RLPA-1* and a sustained expression of *RLPA-2*, which is in agreement with the predominant expression of *RLPA-2* in adenocarcinomas (Shida *et al*, 2004). Whether sPLA₂-III plays a role in the production of LPA, which in turn activates RLPA-2, remains to be established.

The sPLA₂-III as a novel potential biomarker of human colon cancer

Because of their upregulation during colorectal carcinogenesis, Wnt target genes and their associated products have been examined for their potential use as biomarkers. The level of *Il-8* protein was found to be increased in colorectal cancer patients (Terada *et al*, 2005). A significant increase in the level of defensin $\alpha 6$ has also been found in patients with colon cancer (Nam *et al*, 2005). Although *Il-8* and defensin $\alpha 6$ levels have been recently proposed as markers of human colorectal cancer (Nam *et al*, 2005; Terada *et al*, 2005), our data show that they would detect only left-sided adenocarcinomas (Figures 3–5). In contrast, the increased expression of sPLA₂-III was observed in both left and right colon adenocarcinomas, indicating that the analysis of sPLA₂-III levels would detect cancers located in both left and right colon. *MMP-7* is a matrix metalloprotease that has been associated with tumour invasion and metastasis (Nelson *et al*, 2000). We found that the expression pattern of *PLA2G3* was more similar to that of *MMP-7*, which was also increased in both left and right colon adenocarcinomas (Figures 3–5).

In conclusion, this work is the first comprehensive analysis of the expression pattern of the full set of sPLA₂s in human colon cancer. The distinct expression pattern observed for sPLA₂ genes suggests that mRNA profiling of the full set of human sPLA₂s may be useful to detect colon tumours either by analysing their expression pattern in tumours (Barrier *et al*, 2005), in circulating blood cells (Burczynski *et al*, 2005) or directly in serum on circulating mRNA, as recently proposed for other genes (Li *et al*, 2006). Moreover, we have observed a dramatic increase in sPLA₂-III expression in both left- and right-sided adenocarcinomas, suggesting that sPLA₂-III may represent a novel broad molecular biomarker of colon cancer. It will be of interest to determine whether the expression level of sPLA₂-III also increases at earlier stages of tumorigenesis, including adenomas. It will also be useful to detect the sPLA₂-III protein in human colon biopsies, stools or serum using the recently time-resolved fluoroimmunoassays developed for the different human sPLA₂s (Nevalainen *et al*, 2005).

ACKNOWLEDGEMENTS

We thank Mrs Sylvie Dumont for helping in IHC experiments, the Cancer Est Tissue Bank for providing some of the tissue samples and Dr Glenn Belinsky for preparing some colon tumour

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- specimens. Dr Bernard Mari and Dr Claude Auriault are greatly acknowledged for fruitful discussions. This work was supported in part by the Centre National de la Recherche Scientifique (CNRS) to GL, the Association pour la Recherche sur le Cancer (ARC) to GL and the NIH Grant CA-114635 to DWR.
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