PLA2 (group IIA phospholipase A2) as a prognostic
determinant in stage II colorectal carcinoma

A. Buhmeida1*, R. Bendardaf1, M. Hilska2, J. Laine3, Y. Collan3, M. Laato2, K. Syrjänen1 &
S. Pyrhönen1

Departments of 1Oncology and Radiotherapy, 2Surgery and 3Pathology, Turku University Hospital, University of Turku, Turku, Finland

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Background: Approximately 30% of all colorectal cancer (CRC) patients are diagnosed with stage II disease. Adjuvant therapy is not widely recommended. However, it is well established that a subgroup of patients with stage II are at high risk for recurrence within their lifetime and should be considered for adjuvant chemotherapy. The present work was designed to assess the value of group IIA phospholipase A2 (PLA2) as a predictor of disease outcome in stage II CRC patients with long-term follow-up.

Patients and methods: The present study comprises a series of 116 patients who underwent bowel resection for stage II CRC during 1981–1990 at Turku University Hospital. Archival paraffin-embedded CRC tissue samples were used to prepare tissue microarray blocks for immunohistochemical staining with PLA2.

Results: Fifty-five percent of all tumors were positive for PLA2. There was no significant correlation between PLA2 expression and age, sex, depth of invasion and lymph node status. In Kaplan–Meier survival analysis, there was a significant (P = 0.010) difference in disease-free survival (DFS) between patients with negative tumors (longer DFS) and those with positive tumors. The same was true with disease-specific survival (DSS), patients with PLA2-negative tumors living significantly longer (P = 0.025). In multivariate (Cox) survival analysis, however, PLA2 was not an independent predictor of DFS or DSS. In subgroup analysis, the right-sided tumors with negative PLA2 staining had remarkably better prognosis (P = 0.010) than PLA2-positive left-sided tumors.

Conclusions: Quantification of PLA2 expression seems to provide valuable prognostic information in stage II CRC, particularly in selecting the patients at high risk for recurrent disease who might benefit from adjuvant therapy.

Key words: adjuvant therapy, localization, PLA2 expression, stage II CRC, survival

introduction

Molecular genetics of colorectal cancer (CRC) is one of the most intensively studied subjects in human molecular pathology. Abnormalities reported involve tumor suppressor genes that undergo inactivation (e.g. APC, MCC, DCC, p53 and possibly genes in chromosomes 8p, 1p and 22q) and the oncogenes ras, src and myc [1]. Most CRCs seem to arise from adenomas through a multistage process. Leister et al. [2] reported that in human CRC cells, there is a high frequency (42%) of deletions in the short arm of chromosome 1, region 1p35. Bardi et al. [3] have found genetic alterations in chromosome 1p36 in dysplastic colonic adenomas. Alterations of chromosome 1p have also been detected in human neuroblastomas [4], breast cancers [5], mesotheliomas [6] and melanomas [7]. The gene of human group IIA phospholipase A2 (PLA2) has been mapped to chromosome 1p35–36 [8].

PLA2 is an enzyme that catalyzes the hydrolysis of the fatty acyl ester bond at the sn-2 position of phospholipids to produce free fatty acids and lysophospholipids. In human tissues, PLA2 is present in several cell types and different forms of the enzyme are divided into groups according to the structure of the enzyme molecule [9]. Group IIA PLA2 is a 14-kDa enzyme found in a number of tissues and secretory products [10]. The plasma concentration of the enzyme increases dramatically in severe infections and other diseases involving generalized inflammation and cancer [11]. In the gastrointestinal tract, expression of group IIA PLA2 has been localized in Paneth cells of the small intestine [12], metaplastic Paneth cells of gastric [12] and colonic mucosa [13] as well as columnar epithelial cells of inflamed colonic mucosa. Kennedy et al. [14] found overexpression of group IIA PLA2 protein and messenger RNA in colorectal adenomas of patients with familial adenomatous polyposis (FAP). However, the localization and biological role of the enzyme in colonic adenomas and carcinomas remains unclear.

PLA2 is a key regulatory enzyme in arachidonic acid metabolism, which leads to synthesis of prostaglandins via the
cyclooxygenase (COX-1 and COX-2) pathways [15]. Inhibition of COX-2 results in suppression of tumor development and growth, and it has been proposed that prostaglandins may have an important role in tumor growth in CRC [16–18]. Sheng et al. [19] reported that prostaglandin E2 induced bcl-2 gene expression and inhibited apoptosis in human colorectal cell lines in vitro, which may partially explain the COX-2-mediated tumor growth. Thus, in addition to cyclooxygenases, functional defects in PLA2 in tumor cells may interfere with the regulatory mechanisms of tumor growth. Group IIA PLA2 gene has been proposed as a candidate target for Mom1 in the mouse. However, the association between the mutations in the gene coding group IIA PLA2 and the development of FAP [20] or colon carcinoma [21] has been questioned later. Moreover, mutations have not been found in the group IIA PLA2 gene in human cancer cell lines [22]. The PLA2 content of CRC is much higher than the content of normal colorectal mucosa [23]. The data of subcutaneously injected cancer cells also support the importance of PLA2 in association with CRC [24]. Approximately 30% of all CRC patients are diagnosed with stage II disease (node-negative patients) [25]. However, it is well established that a subgroup of patients (20%–25%) with stage II disease are at high risk for recurrence [26] and should be considered as candidates for adjuvant chemotherapy [27]. However, the decision to use adjuvant therapy after curative surgical resection of stage II CRC is often difficult [28] and its routine use is not recommended [29]. There is hope, however, that these decisions could be made more rationally in the future, as soon as more solid data are available on disease predictors in these patients, e.g. molecular markers to disclose subgroups of patients eligible for adjuvant therapy.

As part of our systematic search for prognostic factors in CRC, the present study was conducted to assess the prognostic value of group IIA PLA2 in patients with stage II CRC.

**patients and methods**

**patients and their samples**

The present study comprises a series of 116 patients who underwent bowel resection for stage II CRC during 1981–1990 at Turku University Hospital (Turku, Finland). All patients had been subsequently followed up at regular clinical visits until death or when last seen alive (end of 2005), for a mean of 122 months (range 11.6–263.0 months). The paraffin blocks of the tumor samples were collected from the archives of the Department of Pathology, University of Turku. All pertinent clinical and histopathological data of the patients and their tumors were collected from the patients’ case records. The key clinicopathological data of the patients are summarized in Table 1.

**tissue microarray**

Archival paraffin-embedded CRC samples were used to produce tissue microarray (TMA) blocks for immunohistochemical (IHC) staining. Areas of invasive tumor with the lowest degree of differentiation and rich in atypia, rich in cells, with the highest number of mitoses were chosen. Wide and homogenous areas (compatible with cellular atypia and poor differentiation) were preferred. Necrotic and autolytic areas and areas rich in stromal reaction were avoided. For tumors producing abundant intra- or extracellular mucin, invasive areas with the highest number of epithelial cells were chosen. These representative areas of the tumor were marked by an experienced pathologist (JL) on slides stained with hematoxylin and eosin from selected paraffin blocks, and a cylinder of tissue 1 mm in diameter was cut (MH) with a TMA instrument (Beecher Instruments, Sun Prairie, WI) into a new paraffin block. This size of tissue section (1 mm wide) was equal to the often used three cores, 0.6 mm wide [30–33]. Because the core was larger than usual, sampling differences were less than in 0.6-mm cores. Serial 4-µm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate™ Capillary Gap plus Slides (Gray) by DAKO (Glostrup, Denmark). Normal colorectal mucosa was selected adjacent to but at least 2 mm apart from the malignant growth of the section. If available, another normal sample was obtained from normal colorectal mucosa at either of the resection margins in the surgical specimens. So, usually two normal controls were available. Lymphatic follicles and hyperplastic and inflamed areas were avoided. To obtain enough mucosa for tissue array, tangentially cut areas were avoided.

**immunohistochemistry**

PLA2 protein was stained immunohistochemically using automated staining system, DAKO Autostainer (DAKO). TMA sections were first stained with tissue microarray (TMA) blocks. The sections were then mounted on ChemMate™ Capillary Gap plus Slides (Gray) by DAKO (Glostrup, Denmark). Normal colorectal mucosa was selected adjacent to but at least 2 mm apart from the malignant growth of the section. If available, another normal sample was obtained from normal colorectal mucosa at either of the resection margins in the surgical specimens. So, usually two normal controls were available. Lymphatic follicles and hyperplastic and inflamed areas were avoided. To obtain enough mucosa for tissue array, tangentially cut areas were avoided.

**Table 1. The association of PLA2 expression (no expression/positive expression) with the clinical variables**

<table>
<thead>
<tr>
<th>Clinicopathological feature</th>
<th>No. of patients (%)</th>
<th>PLA2 positive, n (%)</th>
<th>PLA2 negative, n (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>54 (47)</td>
<td>24 (44)</td>
<td>30 (56)</td>
<td>0.710a</td>
</tr>
<tr>
<td>&gt;65</td>
<td>62 (53)</td>
<td>31 (50)</td>
<td>31 (50)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>48 (41)</td>
<td>26 (54)</td>
<td>22 (46)</td>
<td>0.520a</td>
</tr>
<tr>
<td>Female</td>
<td>68 (59)</td>
<td>29 (43)</td>
<td>39 (37)</td>
<td></td>
</tr>
<tr>
<td>Primary tumor status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>90 (78)</td>
<td>45 (50)</td>
<td>45 (50)</td>
<td>0.374a</td>
</tr>
<tr>
<td>T4</td>
<td>26 (22)</td>
<td>10 (39)</td>
<td>16 (61)</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>15 (13)</td>
<td>6 (40)</td>
<td>9 (60)</td>
<td>0.066b</td>
</tr>
<tr>
<td>Grade II</td>
<td>87 (75)</td>
<td>46 (53)</td>
<td>41 (47)</td>
<td></td>
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<tr>
<td>Grade III</td>
<td>14 (12)</td>
<td>3 (21)</td>
<td>11 (79)</td>
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<tr>
<td>Localization of primary tumor</td>
<td></td>
<td></td>
<td></td>
<td>0.007a</td>
</tr>
<tr>
<td>Right colon</td>
<td>45 (39)</td>
<td>14 (31)</td>
<td>31 (69)</td>
<td></td>
</tr>
<tr>
<td>Left colon</td>
<td>35 (30)</td>
<td>20 (57)</td>
<td>15 (43)</td>
<td></td>
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<td>Rectum</td>
<td>36 (31)</td>
<td>21 (58)</td>
<td>15 (42)</td>
<td></td>
</tr>
<tr>
<td>Recurrence during follow-up</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>42 (36)</td>
<td>27 (64)</td>
<td>15 (36)</td>
<td>0.007a</td>
</tr>
<tr>
<td>No</td>
<td>74 (64)</td>
<td>28 (38)</td>
<td>46 (62)</td>
<td></td>
</tr>
<tr>
<td>10-year disease-specific survival</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Alive</td>
<td>43 (32)</td>
<td>15 (35)</td>
<td>28 (65)</td>
<td>0.014a</td>
</tr>
<tr>
<td>Dead</td>
<td>39 (28)</td>
<td>25 (64)</td>
<td>14 (36)</td>
<td></td>
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<tr>
<td>Status at the end of follow-up</td>
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<td></td>
<td></td>
<td>0.050a</td>
</tr>
<tr>
<td>Alive</td>
<td>41 (35)</td>
<td>14 (34)</td>
<td>27 (66)</td>
<td></td>
</tr>
<tr>
<td>Dead</td>
<td>75 (65)</td>
<td>25 (64)</td>
<td>45 (34)</td>
<td></td>
</tr>
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</table>

Significant associations are bolded.

*aChi-square test (likelihood ratio statistic).

bFisher’s exact test.
treated with a polyclonal rabbit anti-group IIA PLA2 antibody [34]. The immunoreaction was localized by using a Vectastain avidin–biotin–peroxidase complex kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer’s instructions. In negative controls, the primary antibody was replaced by preimmune rabbit serum. After staining, the sections were dehydrated in ethanol, cleared in xylene and covered with Mountex and coverslips.

evaluation of PLA2 staining
The expression of PLA2 in the tumor tissue and in the surrounding stromal tissue was assessed by two observers (RB and AB) blinded to the clinical data. In case of discrepant recording, a consensus score was used. The evaluation of staining of all TMAs was done with a Nikon light microscope at the magnification of ×40 without knowledge of tumor grade, stage or clinical outcome. The stromal tissue was invariably negative for PLA2.

Three different grading (A, B and C) systems were applied to assess the cytoplasmic PLA2 expression in tumor cells. In system A, the cytoplasmic staining was graded into four categories: (0) no expression, no detectable staining; (1) weak but detectable staining; (2) moderate, clearly positive staining and (3) intense staining [35]. In system B, cytoplasmic staining was graded in two categories: (i) no/weak expression and (ii) moderate/strong expression. Finally, in grading C, PLA2 expression was categorized simply as no expression or positive. All three systems were statistically tested, and the negative/positive grading (C) seemed to provide the best correlates of PLA2 with the clinically important data.

statistical analysis
Statistical analyses were carried out using the SPSS® for Windows, version 16.0.2 (SPSS, Inc., Chicago, IL), and STATA/SE 10.1 (Stata Corp., College-Station, TX) software packages. Frequency tables were analyzed using the chi-square test, with likelihood ratio or Fisher’s exact test to assess the significance of association between the variables. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using nonparametric tests (Mann–Whitney U test or Kruskal–Wallis test) for two and multiple independent samples, respectively. Analysis of variance was only used for deriving the mean values (and their 95% CIs) of each individual stratum. Univariate survival analysis for the outcome measure [disease-specific survival (DSS), disease-free survival (DFS)] was based on the Kaplan–Meier method, with log-rank (Mantel–Cox) comparison test. To assess the value of PLA2 as an independent predictor, a multivariate survival analysis was done, using the Cox proportional hazards regression model controlling for the confounding of PLA2 by the following variables: age, sex, tumor localization, T and grade (for DFS) and recurrence as additional variable (for DSS). In all tests, the values with $P < 0.05$ were regarded as statistically significant.

results
general description of PLA2 expression
The staining patterns of PLA2 in CRC lesions are illustrated in Figure 1. In the normal colonic mucosa, the staining for PLA2 varied from negative to granular staining localized at the basal and/or apical part of the epithelial cells. Cancer cells also showed granular cytoplasmic staining, with same variation in the staining intensity from gland to gland within the same sample. The cause of variation in normal (control) samples remained unclear, but the results suggested that control samples from the immediate neighborhood of the neoplasm more often showed positivity than the control samples from faraway resection margins.

PLA2 expression correlates with the clinicopathological features
Age, sex or tumor stage had no significant relationship with the expression of PLA2 (Table 1). Higher tumor grade was associated with less PLA2 expression at borderline significance ($P = 0.066$). As to the localization of the tumor, there was a clear difference between tumors situated on the right (caecum and ascending and transverse colon) and on the left (descending and sigmoid colon and rectum) colorectum ($P = 0.007$). On the right side, 69% of the cases were completely negative for PLA2, compared with 42% of the left-sided tumors.

The stromal tissue was not stained. Fifty-five percent of all tumors were positive for PLA2, i.e. 55% showed positive epithelial staining.

Figure 1. Different immunohistochemical staining patterns for phospholipase A2 (PLA2) in cancer epithelium of stage II colorectal carcinoma. A medium-power microscopic view of adenocarcinoma showing different cytoplasmic expressions (no expression and positive expression).
survival analysis

PLA2 expression showed a significant correlation with disease recurrence. A total of 64.3% of patients developing tumor recurrence had PLA2-positive original tumor, as compared to only 37.8% patients with positive expression and had no subsequent recurrence (OR 2.95, 95% CI 1.34–6.49; \(P = 0.007\)).

In Kaplan–Meier survival analysis, there was a significant (\(P = 0.010\)) difference in DFS between patients with negative tumors (longer DFS) and those with positive tumors (Figure 2). The same was true with DSS (Figure 3), patients with PLA2-negative tumors living significantly longer (\(P = 0.025\)), including the 10-year DSS (\(P = 0.014\)).

The role of PLA2 as an independent predictor of DFS was assessed in a multivariate survival (Cox) analysis, containing age, sex, tumor localization, T and G as other variables. In this multivariate model, the only independent predictor of DFS was tumor localization (=colon), Hazard Ratio (HR) 3.149 (95% CI 1.649–6.014; \(P = 0.001\); rectum as the reference). All other variables were removed from the model in stepwise backward approach. When the same model was used to assess the role of PLA2 as an independent predictor of DSS (with disease recurrence as additional variable), PLA2 was not an independent predictor. Not unexpectedly, the only independent predictors of DSS were (i) disease recurrence (\(P = 0.0001\)), with HR 133.857 (95% CI 17.182–1042.801) for dying of disease among patients with disease recurrence and (ii) age (\(P = 0.042\); HR 1.03, 95% CI 1.001–1.063). When recurrence was omitted from the model, tumor localization was the only independent predictor of DSS, HR 2.129 (95% CI 1.120–4.047; \(P = 0.021\); rectum as the reference).

In further analysis, a subgroup of right-sided tumors with negative PLA2 staining had significantly (\(P = 0.08\)) more favorable prognosis than PLA2-positive left-sided tumors (Figure 4). The difference in 5-year survival rate was 45%, and by the 10-year follow-up, 27 of 41 (66%) patients with left-sided, PLA2-positive tumors had died of disease. Right-sided PLA2-positive neoplasm and left-sided PLA2-negative neoplasm had about the same prognosis. This suggested that location and PLA2 staining had about the same influence on prognosis. The curves (Figure 4) further suggested that PLA2 expression was better in prognostication than location of the neoplasm.

discussion

Only a small amount of group IIA PLA2 has been found in normal colonic mucosa [12]. However, increased expression of group IIA PLA2 is present in the colonic mucosa of patients with chronic inflammatory bowel disease [13, 36, 37] and in normal mucosa adjacent to CRC [38]. The present study is the first to systematically assess the expression of group IIA PLA2 in human CRC, as related to clinical data and disease outcome. In our series, normal colonic epithelium often showed expression of PLA2 but lamina propria did not show any expression of PLA2. The positive epithelial staining seemed to reflect the pattern that the epithelium stained when adjacent to CRC [38]. Altogether, 55% of the 116 carcinomas showed positive epithelial cytoplasmic PLA2 expression.
Other studies on CRC have reported overexpression of cytoplasmic PLA2 in tumor cells up to 35%–50% [39–41]. Wendum et al. [42] showed a high expression of cytoplasmic PLA2 in the superficial stroma, just below the surface epithelium and identified these cells as fibroblasts or myofibroblasts. However, none of these studies have correlated PLA2 expression with prognosis of CRC.

In our study, interesting correlations were found between the PLA2 expression and some important clinicopathological parameters. First, a borderline inverse association was shown between PLA2 and tumor grade, low expression of PLA2 being more common among high-grade tumors. This suggests the role of PLA2 as a biological factor that might affect the behavior of the tumor cell population. In this series, the majority (75%) of cases were grade 2 lesions, however, precluding the reach of statistical significance, which remains to be confirmed in a larger study.

Interestingly, we observed a close association between PLA2 and tumor localization; negative expression of PLA2 was significantly associated with right-sided (proximal) tumors of the colon. This was also clearly associated with different long-term survival of these two groups (Figure 4). This suggests that there may be differences between normal right and left colonic segments that could favor malignant transformation through different molecular pathways. Such differences are probably related to different molecular profile of the tumors, microsatellite instability and methylator phenotypes being associated with right-sided tumors and chromosomal instability with the left-sided tumors [43, 44]. We suggest that the higher levels of PLA2 expression associated with distal tumors may be due to these divergent genetic pathways present in the left-sided and right-sided tumors. However, this remains only speculative at this stage and future molecular studies are necessary to confirm this hypothesis [45–47].

Indeed, the role of group IIA PLA2 in the pathophysiology of premalignant cells and the enzyme’s possible role in carcinogenesis, when premalignant cells evolve to malignant phenotype, are unclear. It has been reported that PLA2 inhibitors induce apoptosis in human endothelial cells [48], whereas inhibition of secretory (group IIA) PLA2 in rat intestinal and mouse colon cancer cell lines both induces proliferation and inhibits apoptosis by an unknown mechanism [49]. Interestingly, in the present study, positive expression of PLA2 was a sign of ominous disease outcome in terms of shorter DFS and DSS (Figures 2 and 3). This suggests that PLA2 expression and some important clinicopathological parameters will hopefully lead to practical clinical implementations.

References


