Reduced expression of a gene proliferation signature is associated with enhanced malignancy in colon cancer

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The association between cell proliferation and the malignant potential of colon cancer is not well understood. Here, we evaluated this association using a colon-specific gene proliferation signature (GPS). The GPS was derived by combining gene expression data obtained from the analysis of a cancer cell line model and a published colon crypt profile. The GPS was overexpressed in both actively cycling cells in vitro and the proliferate compartment of colon crypts. K-means clustering was used to independently stratify two cohorts of colon tumours into two groups with high and low GPS expression. Notably, we observed a significant association between reduced GPS expression and an increased likelihood of recurrence (P <0.05), leading to shorter disease-free survival in both cohorts. This finding was not a result of methodological bias as we verified the well-established association between breast cancer malignancy and increased proliferation, by applying our GPS to public breast cancer data. In this study, we show that reduced proliferation is a biological feature characterizing the majority of aggressive colon cancers. This contrasts with many other carcinomas such as breast cancer. Investigating the reasons underlying this unusual observation may provide important insight into the biology of colon cancer progression and putative novel therapy options.

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Defective regulation of cell proliferation is a fundamental feature of cancer. By providing a genome-wide insight, microarray technology has revealed that conserved tumour expression patterns include many proliferation-associated genes termed as ‘proliferation signatures’. In most cancers, an increased expression of proliferation signatures has been associated with enhanced malignancy (Rosenwald et al, 2003; Krasnoselsky et al, 2004; Dai et al, 2005) suggesting cell proliferation as a driving force for cancer progression. Breast cancer is a typical example where the hyper-proliferative nature of more aggressive subtypes has been confirmed by array-based proliferation assays (Sorlie et al, 2001; Dai et al, 2005). These studies have also proved the superiority of cell proliferation analysis by microarrays over conventional methods regarding its objectivity and because of the assessment of multiple genes (Paik et al, 2004; Glinkys et al, 2005; Whitfield et al, 2006).

In colorectal cancer (CRC), the impact of tumour proliferation rate on malignancy is unclear because the conventional proliferation markers have produced conflicting results (Supplementary Figure 1). To date, no proliferation signature has been established to address such an association.

To investigate the association between proliferation and aggressiveness of colon cancer, we designed a colon-specific gene proliferation signature (GPS) that was derived from common expression patterns of an in vitro CRC model, and of human colon crypt compartments as published by Kosinski et al (2007). The GPS was applied to evaluate two independent cohorts of colon tumours and revealed an inverse relationship between tumour cell proliferation and unfavourable clinico-pathological parameters.

MATERIALS AND METHODS

Cell cultures

The following 10 CRC cell lines were used: DLD-1, HCT-8, HCT-116, HT-29, LoVo, LS174T, SK-CO-1, SW48, SW480 and SW620 (ATCC, Manassas, VA, USA). Cells were cultivated in α-MEM supplemented with 10% foetal bovine serum, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (GIBCO-invitrogen, CA, USA). Two cell cultures were established for each cell line for RNA preparation. The first culture was harvested at semi-confluence (50–60%) when cells were actively cycling. Cells in the second culture were harvested 48 h after reaching full confluence, when cells were slow-growing. A replicate experiment was performed to obtain RNA for dye-reversed hybridisations.

Patients

Tumours from two cohorts of colon cancer patients were analysed. Patient follow-up was a minimum of 5 years. Disease-free survival
A proliferation signature in colon cancer

A Anjomshoaa et al.

A proliferation signature in colon cancer patients and associations with the GPS

Table 1 Clinico-pathologic characteristics of two cohorts of colon cancer patients and associations with the GPS

| Parameters                      | Numbers | Cohort A | Cohort B | Cohort A (P-value)* | Cohort B (P-value)* |
|---------------------------------|---------|----------|----------|---------------------|---------------------|
| Age<sup>a</sup>                 |         | < Mean 49 | 20       | 0.33                | 0.79                |
| Age> Mean                      |         | 59       | 17       |                     |                     |
| Sex                             |         | Male 62  | 21       | 0.56                | 0.74                |
| Sex                             |         | Female 46 | 16       |                     |                     |
| Vascular invasion               |         | Yes 9    | 1        | 0.065               | NA                  |
| Vascular invasion               |         | No 99    | 36       |                     |                     |
| Lymphatic invasion              |         | Yes 23   | 3        | 0.018               | 1                   |
| Lymphatic invasion              |         | No 85    | 34       |                     |                     |
| Margins                        |         | Infiltrative 56 | NA | 0.84               | 0.19                |
| Margins                        |         | Expansive 52 |       |                     |                     |
| Chemotherapy                    |         | Yes 30   | 0        | 0.08                | NA                  |
| Chemotherapy                    |         | No 78    | 37       |                     |                     |
| Recurrence at 5 years           |         | Yes 24   | 16       | 0.021               | 0.005               |
| Recurrence at 5 years           |         | No 84    | 21       |                     |                     |
| Total                           |         | 108      | 37       |                     |                     |

Abbreviations: GPS = gene proliferation signature; NA = not applicable.

*<sup>a</sup>A Fisher’s Exact test or Kruskal–Wallis test were used for testing association between clinico-pathologic parameters and the dichotomous GPS variable.

<sup>a</sup>Average age 68 and 63 years for cohort A and B patients, respectively. Bold numbers represent significant P-values.

The expression of seven randomly selected genes from the GPS (MAD2L1, POLE2, CDC2, MCM6, MCM3, TOPK and GMNN) was validated by quantitative real-time PCR (qRT–PCR) on an ABI Prism 7900HT Sequence Detection System using cell line cDNA and Taqman Gene Expression assays (Applied Biosystems, CA, USA). Relative fold changes were calculated using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001) with Topoisomerase 3A as the internal control. Reference RNA was used as the calibrator to enable comparison between different experiments.

Immunohistochemical analysis

Ki-67 immunohistochemistry was performed on 40 cohort A tumours for which paraffin blocks were available, and an additional set of 33 rectal/rectosigmoid tumours was included to increase statistical power. Antigens were retrieved on 4-µm sections in boiling citrate buffer (pH 6). Primary
antibody (MIB-1, DakoCytomation, Denmark; dilution 1:50) was detected using the EnVision system (Dako EnVision) and the DAB substrate kit (Vector Laboratories, CA, USA). Five high-power fields were counted by two observers in a blinded manner. The Ki-67 proliferation index (PI) was presented for each tumour as the percentage of positively stained nuclei.

**Statistical analysis**

The K-means clustering method was applied to stratify clinical samples on the basis of the GPS expression level using the TIGR MeV 4.0 software (Saeed et al, 2003). Using Pearson uncentered correlation, tumours from each cohort were assigned to two clusters (i.e., K-means clustering with \( K = 2 \), 1000 iterations of clustering) with the threshold of occurrence in the same cluster set to 80%. The consensus clusters each contained tumours with similar GPS expression, resulting in two patient groups differing in their GPS levels. Either Fisher’s exact test or the Kruskal–Wallis test was then used to evaluate the associations between the dichotomous GPS variable and clinico-pathologic parameters. Statistical analyses were performed using SPSS 15.0.0 (SPSS Inc., Chicago, IL, USA). For Ki-67 analysis, tumours were stratified into two clusters with the mean Ki-67 value as a cutoff point.

The gene set comparison function from the BRB-Array tools software was used to analyse the GPS for differential expression among subgroups defined by the clinico-pathologic variables. The GPS was considered to have a higher-than-expected number of genes differentially expressed (DE) between the classes being compared if the Kolmogorov–Smirnov (KS) resampling \( P \)-value was less than 0.005 (default value). The distribution of KS statistics was obtained by 100,000 iterations of the random resampling process.

Disease-free survival was plotted using the method of Kaplan and Meier, and a log-rank test was used to test for differences in survival time between defined clusters of patients according to the GPS or Ki-67 PI. A multivariate Cox proportional hazards model was developed using forward stepwise regression with predictive variables that were significant in the univariate analysis. Cox multivariate regression was not relevant for measuring the performance of the GPS in cohort B, as this population of tumours included only stage II colon cancers.

**RESULTS**

**Derivation of a GPS**

To identify a set of genes whose expression was associated with tumour cell proliferative activity, two proliferation-based systems were analysed and integrated as described below and illustrated in Figure 1.

**A. Gene expression analysis of a CRC in vitro model**

The in vitro system involved identification of genes that were to reflect the proliferative activity of CRC cell lines. Genes DE between exponentially growing (nonconfluent) and growth-inhibited (confluent) cell lines were identified (Figure 1A–C). Firstly, DE genes between Cy5-labelled nonconfluent and confluent samples were identified by statistical analysis of microarray data (two-class paired, FDR < 1%; Tusher et al, 2001). Secondly, DE genes between Cy3-labelled nonconfluent and confluent samples (biological replicates) were identified using the same approach. To minimise gene-specific dye bias and other sources of variation, only genes that were present in both SAM-generated gene sets were selected. The gene set was further reduced to genes whose expression was consistently altered in the same direction in at least 8 out of 10 cell lines, yielding a total 881 DE genes with known annotation (Figure 1D).

Gene ontology (GO) analysis showed that categories related to cell cycle and DNA metabolism were the most over-represented biological themes among the DE genes, mainly because of genes that were overexpressed in exponentially growing cells \( (P < 10^{-5}) \).

**B. Gene expression profile of the proliferative compartment of colon crypts**

The second gene set used for the design of a GPS was based on the physiological expression profile of human colon crypts. Kosinski et al (2007) compared the proliferative bottom part of crypts with the differentiated crypt top, and identified 299 DE genes highly expressed in the proliferative bottom (Figure 1E). The GO terms that were over-represented within this gene list were related to cell proliferation and renewal, consistent with the physiological function of the bottom crypt compartment.

**C. Definition of the GPS**

To define a final GPS enriched in key-proliferative genes, the in vitro and in vivo-derived DE gene lists were screened for common genes (Figure 1F). Thirty-six genes were found to be overexpressed in both exponentially growing CRC cell lines and the proliferative crypt compartment. These genes were defined as GPS that included 15 cell cycle-related genes (Supplementary Figure 2).

The expression of 7 genes randomly selected from the 15 cycle-related genes was validated by qRT–PCR on the cell line cDNAs. A close correlation between qRT–PCR and array data was observed (Supplementary Table 2).

**Classification of colon tumours according to the GPS expression level**

To investigate the association between the GPS and patient parameters, expression values of the GPS genes were first obtained from the array-generated expression profiles. Expression data were available for all 36 genes in cohort A and 35 genes (except CDCAS3) in cohort B tumours. For each cohort, tumours were split into two consensus clusters on the basis of their GPS expression using K-means clustering. Tumours with relatively high or low GPS expression were defined as having high or low proliferative activity, respectively (Figure 1G).

**Reduced expression of the GPS is associated with unfavourable clinico-pathologic variables**

Intriguingly, we observed an association between reduced GPS expression and an increased risk of recurrence in both cohorts (Table 1). Groups with reduced GPS expression were significantly enriched for recurrent tumours \( (P = 0.021 \text{ and } 0.005 \text{ for cohort A and B, respectively}) \). In cohort A, reduced GPS expression was also associated with higher disease stage \( (P = 0.015) \). Further, reduced GPS expression was significantly more frequent in cohort A tumours with lymphatic invasion compared with those without lymphatic invasion \( (P = 0.018) \). Gene set comparison analysis confirmed that GPS contained a higher-than-random proportion of DE genes among subgroups with clinico-pathologic parameters for which a significant association with the GPS was found in nonparametric tests (Supplementary Table 1).

**Reduced GPS expression is associated with shorter DFS in colon cancer**

To examine whether a difference in cell proliferation determined by the GPS may be associated with time to recurrence, DFS was plotted for low GPS and high GPS tumours (Figure 2). DFS was significantly shorter in patients with reduced GPS expression \( (P = 0.033 \text{ and } 0.011 \text{ for cohort A and B patients, respectively}; \text{ Figure 2A and C}) \). This association remained significant in cohort
A, when patients with adjuvant therapy were excluded ($P=0.029$; Figure 2B).

If proliferative activity is a crucial factor influencing DFS, then a GPS consisting of only genes directly implicated in cell cycle process should be sufficient to separate patients into groups with different survival rates. Therefore, the GPS was reduced to the 15 cell cycle-regulated genes and survival analysis was repeated. The modified GPS stratified patients into groups that differed in their DFS more stringently ($P=0.022$ and $0.003$ for cohort A and B patients, respectively; Figure 2D–F) than the original GPS including all 36 genes. Therefore, colon cancer patients with tumours of lower proliferative activity are more likely to recur.

When the parameters predicting patient outcome in univariate analysis were investigated in a multivariate model, disease stage was the only independent predictor of 5-year DFS in cohort A (Table 2).

**Increased GPS expression is associated with shorter DFS in breast cancer**

As the association between reduced GPS expression and poor colon cancer prognosis was an unexpected finding, we tested the validity of our GPS on public array data from two independent breast cancer cohorts. Using these data, an association between increased proliferation and bad outcome has been established earlier (van de Vijver et al., 2002; Pawitan et al., 2005).

For each breast cancer data set, patients were stratified into two groups with either low or high GPS expression and their DFS was plotted. In both data sets, patients with increased GPS expression had significantly shorter DFS compared with patients with reduced GPS expression ($P<0.0001$; Figure 3), confirming the previously established association. Therefore, the association between low proliferation and bad colon cancer outcome is not the result of biased methodology.
Ki-67 PI is not associated with outcome

Ki-67 PI ranged from 25 to 96%, with a mean value of 76.3 ± 17.5 and a median value of 81.8%. When these 73 patients were stratified into two groups differing in their GPS expression, a significant difference in DFS was apparent (P = 0.01; Supplementary Table 3). However, when patients were stratified into two groups according to the mean Ki-67 PI, the DFS of the group with a low PI was similar to that with a high PI (P = 0.55; Supplementary Table 3). Furthermore, when analysis was limited to patients with the highest and the lowest Ki-67 values, no statistical difference in DFS was observed (data not shown). No correlation was found between the Ki-67 PI and mean GPS expression of tumours (Spearman R = 0.06; P = 0.63).

**DISCUSSION**

Cancer is regarded as a proliferative disorder, where a selective growth advantage is believed to crucially contribute to its development and progression. Supporting this concept are many studies that have shown an association between poor clinical outcome and high expression of proliferation-associated genes (Krasnoselsky et al, 2004; Dai et al, 2005; Kirschner-Schwabe et al, 2006).

Our study is the first to report an inverse relation between cancer malignancy and the expression of a multi-gene proliferation signature. In two independent cohorts of colon cancer patients, we observed an association between an increased recurrence risk and the reduced expression of a GPS. This finding challenges the
long-held belief that rapidly dividing cancer cells are a harbinger of poor prognosis, and suggests that low proliferative activity is a biological feature of the majority of aggressive colon cancers.

The GPS we designed is highly likely to directly reflect the proliferative state of a tumour, as it was derived from both human colon crypts and an in vitro CRC model. All genes included in the GPS were overexpressed in actively proliferating cells of the both systems. With respect to the in vitro system, the comparison of exponentially growing cancer cells with contact-inhibited cancer cells has limitations; however, well established evidence indicates that many tumour cell lines maintain a variable degree of density-dependant growth suppression that is characteristic of the stationary phase (Couldwell et al., 1992; Steinman et al., 2003; Kuhn et al., 2004; Motti et al., 2005). Ten CRC cell lines were included to ensure an overall growth suppression, and only genes were considered that were altered in at least 80% of the cell lines. By the inclusion of only DE genes that overlapped with the human colon proliferation signature, the final GPS consisted of genes that strongly correlate with both colon cancer cell growth and physiological colon proliferation. Consistent with this, ontology analysis indicated that proliferation-associated genes represent the main biological theme in both the in vitro and the in vivo system.

Further evidence supporting the association of the GPS with cell proliferation stems from a considerable overlap in genes or gene families identified between our GPS and other proliferation signatures defined for tumours of the breast (Perou et al., 1999, 2000), ovary (Welsh et al., 2001), liver (Chen et al., 2002), acute lymphoblastic leukaemia (Kirschner-Schwabe et al., 2006), neuroblastoma (Krasnoselsky et al., 2004), lung squamous cell carcinoma (Inamura et al., 2005), head and neck (Chung et al., 2004), prostate (LaTulippe et al., 2002) and stomach (Hippo et al., 2002). Comparing these published data, Whitfield et al. (2006) identified a core set of genes common to various proliferation signatures. As expected, these genes (MYBL2, PLK1, CDC2 and MCM genes) are also contained within our GPS (see Supplementary Figure 2), reflecting the universal mechanisms that govern human cell division. Indeed, by reanalysis of public breast cancer data, our GPS was shown to perform properly in other cancer types as well. Therefore, our GPS appears to be a reliable tool for the assessment of proliferation in colon cancer.

Application of our GPS to colon cancer patient data revealed a robust association between low proliferative activity and increased likelihood of recurrence. Firstly, the low GPS group had reduced DFS in two independent cohorts derived from different populations. Secondly, expression data from the two cohorts were obtained using two different array platforms, indicating that the observed association was not subject to methodological bias. Thirdly, reduced GPS expression in cohort A also correlated with clinicopathological variables related to poor outcome (stage, lymphatic invasion). A possible confounding factor in our study was the chemotherapy treatment as given in 28% of cohort A patients. Exclusion of these patients from analysis had no effect on the association strength, suggesting that proliferation affects patient outcome independent of adjuvant chemotherpay.

Notably, the observed association was not independent of tumour stage. In other words, higher disease stages were enriched for slowly proliferating tumours, but tumours with high GPS expression were also present. It remains possible, however, that these fast proliferating tumours had progressed slowly before they were diagnosed at an advanced stage. It is likely that tumour stage is a better prognostic factor because the presence of lymph node and distant organ involvement is a direct manifestation of metastasis. It is noteworthy that the goal of this study was not to develop a prognostic tool based on the GPS, but to determine the nature of the relationship between proliferation and the degree of malignancy in colon cancer.

Together, the analyses we performed are all consistent with a marked effect of colon cancer proliferation on the rate of its recurrence. This is an important finding because studies using conventional proliferation markers have not been able to establish a clear-cut association between colon cancer proliferation and outcome. On account of these reported inconsistencies, we also performed DFS analysis on the basis of proliferation estimated by conventional Ki-67 labelling. Unlike the GPS, Ki-67 PI failed to...
separate a set of 73 CRCs into groups associated with distinct survival (Supplementary Table 3). Although the advantage of assessing multiple genes as opposed to one proliferation marker appears obvious, additional parameters are likely to contribute to the inferior performance of the Ki-67 PI. Ki-67 positivity reflects the number of cells in cycle, rather than their cycling speed (Endl and Gerdes, 2000). Furthermore, the visual scoring of Ki-67 positive nuclei is inherently subjective, confounding correlations particularly in the case of small sample sizes (Barzanti et al, 2000). It is of interest, however, that a low Ki-67 PI has been associated with poor outcome in the three studies with the highest statistical power (i.e., largest sample sizes) (Allegre et al, 2003; Garrity et al, 2004; Hilska et al, 2005).

It is difficult to assess from our data whether slow proliferation directly enhances the ability of a colon cancer to metastasise or fast proliferation may be disadvantageous for colon cancers to acquire metastatic capacity. Although it appears intuitively logical that accelerated cell division allows for more genetic events required for progression to accumulate, fast proliferation might also have a negative impact on a tumour ability to survive and spread. For example, rapidly growing tumours may elicit a stronger and more effective immune response. Further, highly proliferative tumours may contain a lower proportion of cancer stem cells thought to undergo relatively slow divisions. Alternatively, a high level of genetic instability may increase the invasive potential of cancer cells or their resistance towards apoptosis, but may interfere with smooth replication. Also, hypoxic conditions may slow down the growth rate of tumours but could promote the onset of epithelial–mesenchymal transitions leading to invasion. Possible underlying mechanisms are currently under investigation in our laboratory.

The intriguing finding of an inverse relationship between tumour proliferative activity and disease aggressiveness suggests that fundamental biological differences exist for the mechanisms that drive disease progression in colon cancer compared with other epithelial malignancies. The delineation of the underlying processes will be important not only for the understanding of colon cancer biology, but also for the design of new therapeutic strategies. In this context, the development of alternative therapies might be a pertinent issue, given that current chemotherapeutic agents are usually designed to target colon cancer progression by killing rapidly proliferating cells.

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