Characterisation of integrin-linked kinase signalling in sporadic human colon cancer

A Marotta¹, K Parhar¹, D Owen², S Dedhar¹,³ and B Salh¹,¹

¹Jack Bell Research Center, 2660 Oak Street, Vancouver, BC, Canada V6H 3Z6; ²Vancouver General Hospital, 855 W12th Ave, Vancouver, BC, Canada V5Z 1M9; ³BC Cancer Agency, 600 W10th Ave, Vancouver, BC, Canada V5Z 4E6

The putative oncogene, integrin-linked kinase (ILK) is a protein serine/threonine kinase that has been reported to regulate a number of biological properties including anchorage-independent cell cycle progression, tumour cell invasion and apoptosis. Overexpression of ILK has been documented in a wide variety of human malignancies including Ewing’s sarcoma (ES), primitive neural ectodermal tumours (PNETs) and prostate tumours (PT). We recently reported that ILK signalling was also dysregulated in patients with the genetic condition familial adenomatous polyposis (FAP), a precursor to colon cancer. In this study, we extended our previous work by investigating the ILK-signalling pathway in sporadic human colon cancer and representative lymph node metastases. The data indicate that the ILK protein is significantly hyperexpressed in malignant acini in relation to normal crypts. Moreover, overexpression of ILK not only coincided with increased MBP phosphotransferase activity but as well with effects on downstream targets like GSK3β. Based upon the presented data, we propose that ILK signalling is dysregulated early during the development of human colon cancer, and that selective inhibition of this molecule alone or in combination with the standard therapeutic modality might be a more effective means of treating colon cancer.

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Mutation of the adenomatous polyposis coli (APC) gene is an integral event in the genesis of colorectal cancer (Fearon et al., 1990; Kinzler et al., 1991). Mutation of this gene results in the expression of a C-terminally truncated protein that is unable to form a complex with axin, β-catenin and GSK3β (Behrens et al., 1998; Sparks et al., 1998; Barker et al., 2000; Rowan et al., 2000). Consequently, there is an increase in the cytosolic levels of β-catenin. Stabilisation of the latter is believed to result in its translocation to the nucleus where it binds to the Tcf-4 (T-cell factor) family of transcription factors resulting in the expression of a number of different genes that have been implicated in oncogenesis. These include cyclin D1, c-myc and the matrix metalloproteinase (MMP)-7 (He et al., 1998; Crawford et al., 1999; Shuttman et al., 1999). However, whether mutation of APC alone is sufficient in dysregulating β-catenin signalling or whether additional signals are required for this disruption are currently unclear. In this regard, a prominent nuclear β-catenin signal was documented in cells that overexpress the integrin-linked kinase (ILK), duplicating the events associated with the mutation of APC. Translocation of β-catenin accompanied the activation of Tcf-4-dependent gene transcription (Novak et al., 1998).

The ILK, which was discovered through its interactions with the β1 integrin subunit (Hannigan et al., 1996), has been demonstrated to mediate a plethora of biological events. This putative oncogene has not only been described as an immunohistochemical marker for the identification of Ewing’s sarcoma (ES) and primitive neuroectodermal tumours (PNET), but as well increased expression of the protein has been demonstrated to be inversely related to the 5-year survival rate in prostate cancer (Chung et al., 1998; Graff et al., 2001). In addition to this, we demonstrated that ILK signalling is dysregulated in patients diagnosed with familial adenomatous polyposis (FAP) (Marotta et al., 2001). In the present study, we sought to determine the extent to which this pathway was disrupted in sporadic cases of colon cancer. The results from these studies demonstrate that ILK is hyperexpressed in malignant crypts from both the primary and metastatic lesions. In addition to this, we demonstrate that there was approximately a 2–9-fold increase in ILK immunoprecipitated MBP phosphotransferase activity in relation to normal colonic crypts. Furthermore, we report that changes in ILK activity coincide with changes on downstream targets, primarily GSK3β. Based upon our findings, we conclude that dysregulation of the ILK-signalling nexus is an important early event in the genesis of human colon cancer.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies for ILK (IB/IHC), PKB and GSK3β were kindly provided by Stressgen Biotechnologies Inc. (Victoria, BC, Canada). Monoclonal anti-ILK (IP) and MBP were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA).
Tissue procurement
We obtained a total of 38 cases of human colon cancer through Dr. D. Owen from the Division of Anatomical Pathology at Vancouver Hospital and Health Sciences Centre (VH&HSC). The 38-paired cases were used for biochemical analysis. In all, 16 of the cases, which were utilised in these studies, were selected for immunohistochemical analysis on the basis that lymph node metastases were present. Ethical consent for these studies was obtained from each of the patients and by boards governing research at the University of British Columbia and VH&HSC.

Preparation of human tissue samples
The tissue samples were serially sectioned (3–5 µm in diameter) using a cryostat, and approximately 20 slices were placed in 1 ml of homogenisation buffer containing 20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride and 10 µg ml⁻¹ leupeptin as previously described (Marotta et al, 2001).

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)
Protein samples for immunoblotting were resolved using SDS–PAGE. Proteins were transferred onto the nitrocellulose membrane in a BioRad transfer apparatus and the nitrocellulose membrane was blotted with the appropriate antibody at a dilution of 1:1000 in 0.05% Tween-TBS. The resulting membrane was exposed to ECL film using the BioRad gel-doc apparatus into a TIFF format file. The relative amounts of protein were measured by scanning the film and the results are expressed as the mean ± s.d.

Immunoprecipitation
A total of 400 µg of the appropriate sample was subjected to a preclear step with a nonspecific rabbit IgG antibody preabsorbed to protein A Sepharose for a minimum of 1 h at 4°C. The samples were then centrifuged 10 000 r.p.m. and equal volumes of the supernatant were taken and aliquoted into a new microfuge tube. The lysate was then incubated with 4 µl of the appropriate primary antibody at a pre-determined concentration overnight at room temperature. Following incubation, the sections were rinsed three consecutive times with PBS and then incubated with the appropriate biotinylated secondary antibody for 1 h followed by incubation with peroxidase-labelled streptavidin. AEC substrate was used as the chromagen and the sections were counterstained with haematoxylin. Three observers independently examined all the stained immunoreactive positivity was assessed by uniform red staining.

The staining intensity (weak = 1, intermediate = 2 and strong = 3) was scored by three independent examiners (AM, DO and BS), and the results are expressed as the mean ± s.d.

Statistical analysis
The relative amounts of protein were measured by scanning the film using the BioRad gel-doc apparatus into a TIFF format file. The numerical densitometric values were assigned arbitrary values on a scale of 1–3 for immunohistochemistry. The band densities and the results are expressed as mean ± s.d., with P < 0.05 being considered significant using the Student’s t-test (unpaired, two-tailed).

RESULTS
ILK expression is dysregulated in sporadic cases of colon cancer
A recent report from our laboratory indicated that the expression and activity of ILK is perturbed in polypoid lesions resected from patients diagnosed with the autosomal dominant condition, FAP (Marotta et al, 2001). To determine whether ILK was also dysregulated in sporadic cases of human colon cancer, we examined both the protein expression of ILK by immunohistochemical analysis and the mRNA levels using microarray technology. Immunohistochemical analysis revealed that the protein levels of ILK were dramatically increased in the cancerous acini when compared to the normal adjacent control crypts based on the intensity of the chromagen (Figure 1A; upper panel). This increase in the protein expression of ILK was further apparent at higher magnification (Figure 1A, lower panels). The results are corroborated by the micrographs presented in Figure 1B–G, which represent three separate cases. These results clearly demonstrate that the protein expression of ILK is increased in the cancerous crypts (panels C, E, G) with respect to the adjacent control tissue for each case (panels B, D, F).

To determine whether overexpression of ILK within these lesions was statistically significant, the relative staining intensity for each case was scored. The data thus obtained revealed that the increase in ILK in the cancerous crypts was highly significant (P < 0.0005). There was approximately a three-fold increase in the expression of ILK in the cancerous acini when compared to the normal adjacent tissue (0.75, Figure 3). It is worth noting that although ILK immunoreactivity was observed in the stromal component of both the normal and cancerous lesions, this staining was not taken into account when quantifying the intensity of the...
chromagen for statistical analysis. ILK immunoreactivity within the stromal component of tissue is not an unexpected finding, since this protein is ubiquitously expressed (Li et al., 1999). Analysis of the mRNA levels indicated that there was no significant differences in the ILK mRNA levels between any of the controls and primary lesions analysed (data not shown). Thus, it appears that the increased ILK protein expression likely reflects a change in protein stabilisation as opposed to a change in the level of the message.

**ILK expression in regional lymph nodes**

A number of studies have underscored the importance of ILK in mediating cell migration and invasion. In this regard, over-expression of ILK has been reported to upregulate the levels of MMP-9 in an AP-1-dependent manner. Moreover, treatment with the selective ILK inhibitor (termed KP-SD1) was shown to inhibit MMP-9 promoter activity as well as lead to a reduction in the invasive potential of intestinal and mammary epithelial cells (Troussard et al., 2000).

To characterise the protein expression of ILK in metastatic lesions, 16 cases were selected on the basis that metastatic deposits were present in the regional lymph nodes. The representative results from these studies are presented in Figure 2. In Figure 2A, which represents a control lymph node, a moderate chromagenic signal is present in a small proportion of the lymphocytes as well as the lymphatic vessels (yellow arrow head). In Figure 2B–E, which represents four additional representative cases demonstrating enhanced ILK expression in the cancerous lesions (C, E, G) when compared with the normal control (B, D, F). Staining was performed as outlined in the Materials and Methods section.
ILK in colonic polyps coincided with an increase in the expression of ILK activity is increased in colonic cancers. Compared to the normal colonic crypts (fold in the malignant acini of the regional lymph nodes when expression of ILK is significantly increased approximately four-fold), the relative staining intensity was quantified which the protein expression of ILK was altered during the metastatic process, the relative staining intensity was quantified compared to the control tissue as well as the primary cancer. 

Methods section.

To determine whether ILK activity was similarly affected in human colon cancer, we characterised the biochemical activity in a total of 38 cases (16 cases in which metastatic deposits were present in the regional lymph nodes; an additional 22 cases with no lymphatic invasion). The representative data (Figure 4A) indicate that changes in the expression of ILK (lower panel) coincided with changes in the MBP phosphotransferase activity in the cancerous lesions when compared to the normal adjacent control tissue. Two- to nine-fold increases in ILK activity were evident in 24 out of the 38 cases (63%). To determine whether there was a direct correlation between ILK expression and activity levels, the percentage change in the ILK activity above the control was compared with the percentage change in the ILK expression above the control (see Figure 4C). Although both displayed increased levels, a direct correlation was not apparent between them. It is worth adding that there were no measurable differences in the ILK protein expression between primary tumours with or without positive lymph nodes. Importantly, changes in the expression and activity of ILK appeared to be independent of changes in the protein expression of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. These results are in keeping with a number of reports that have examined the relative expression and activity of Erk1 and Erk2 in the same lesions. 

Since ILK has been shown to regulate GSK3β activity (Delcommenne et al., 1998) as well as modulate the subcellular distribution of β-catenin (Novak et al., 1998), we wanted to determine whether changes in ILK activity correlated with effects on these downstream targets in vivo. For the representative patient (Figure 4B), changes in the MBP phosphotransferase activity appeared to coincide with not only the overexpression of the ILK protein but as well with the stabilisation of β-catenin. Changes in the expression of the latter are not surprising, since approximately 85% of all sporadic colorectal cancers are believed to arise because mutations in the APC gene (Mei et al., 1999). Interestingly, we observed an impressive increase in the phosphorylation of GSK3β at Ser-9 by immunoblotting with a phosphospecific GSK3β Ser-9 antibody as well as by retardation in the electrophoretic mobility.
of the GSK3β protein itself. Phosphorylation of GSK3β at Ser-9 coincides with a decrease in its phosphotransferase activity. These findings consolidate our preliminary data in colonic polyposis (Marotta et al., 2001) and indicate that this dysregulation is a stable reproducible event in multistage colon carcinogenesis. Additionally, elevated expression and activity of ILK appeared to be associated with an increase in the protein expression of Lef-1 (data not shown), a downstream target of ILK (Novak et al., 1998). The expression of GAPDH was assessed to control for protein loading.

There are a number of reports, which indicate that Ser473 of PKB is phosphorylated by an array of protein kinases including Mapkap-2, PDK1/PRK2 and PKC. ILK has also been described as the putative PDK2 that phosphorylates this site on PKB. In this regard, ILK has been demonstrated to phosphorylate Ser473 in vitro and in vivo. Furthermore, modulation of ILK activity with a specific inhibitor (KP-SD1) has been reported to coincide with a decrease in Ser473 phosphorylation as well as modulate the activity of the respective kinase. Transient and stable overexpression of the ILK protein is known to correlate with an increase in not only Ser473 phosphorylation but also the biochemical activity of PKB.

To determine if changes in ILK expression resulted in changes in the phosphorylation status of PKB, we examined Ser473 phosphorylation by immunohistochemical analysis in the 16 cases in which metastatic lesions were present. The results, which are presented in Table 1, indicate that changes in PKB Ser473 phosphorylation occur infrequently in this disease based upon the intensity of the chromagen. These results are further corroborated by evidence indicating that there were no significant differences in the expression of GAPDH, a housekeeping gene, between control and tumour samples.

Table 1  Relative PKB Ser473 phosphorylation staining intensity

| Case number | Control | Primary cancer | Metastatic lesion |
|-------------|---------|----------------|------------------|
| 3           |         |                |                  |
| 4           |         |                |                  |
| 6           | 2       | 2              |                  |
| 8           | 1       |                | 2                |
| 9           |         |                |                  |
| 12          |         |                |                  |
| 15          |         |                |                  |
| 22          |         |                |                  |
| 23          |         |                |                  |
| 25          |         |                |                  |
| 26          | 1       |                | 2                |
| 27          | 2       | 2              |                  |
| 32          | 1       |                |                  |
| 33          |         |                |                  |
| 35          |         |                |                  |
| 38          |         |                |                  |
| T            |         |                |                  |

Tissue sections corresponding to either control, primary cancer or metastatic lesion were stained as outlined in the Materials and Methods section.

Figure 4  ILK signalling is dysregulated in human colon cancer. (A) ILK activity is enhanced in colorectal cancers. Upper panel, ILK MBP phosphotransferase densitometry. Immunoprecipitated ILK MBP phosphotransferase performed in triplicate. Middle panel, anti-ILK immunoblot, examining the protein expression of ILK in the tumour and the corresponding control sample. Lower panel; anti-Erk1-CT immunoblot, examining expression of Erk1 and Erk2 in the control samples vs the corresponding tumour. (B) Effects of ILK on downstream targets. ILK activity is increased in the polyp compared with its respective control (representative autoradiogram). Anti-ILK immunoblot, examining expression of ILK. Anti-β-catenin immunoblot, examining the expression of β-catenin. Anti-P-GSK3β immunoblot, examining phosphorylation status of GSK3β. Anti-GAPDH immunoblot, examining expression of GAPDH. Anti-GAPDH immunoblot, used as an internal control for experiments. The results are representative for the 24 cases, which displayed increases in ILK activity. (C) Correlation between the expression and activity of ILK in colonic tumours. The band intensities were quantitated as outlined in the Materials and Methods section. The values are represented as a percentage change in activity/expression above the corresponding control sample.
differences in immunoprecipitated PKB HH2B phosphotransferase activity between the control and tumour samples analysed (data not shown). These findings in colon cancer are supported by our recent findings in human breast cancer, which demonstrated that there were no statistically significant differences in PKB activity between the control and tumour samples analysed (Salh et al., 2002). To add further support, mammary tumours induced by specific overexpression of ILK correlated with a dramatic increase in the Ser-9 phosphorylation of GSK3β. However, only modest differences in the Ser473 phosphorylation status of PKB were observed in this animal model (White et al., 2001). Thus, it appears that ILK is more likely to regulate GSK3β activity directly in vivo, and dysregulation of this nexus, rather than carcinogenesis, might have an important role in epithelial-derived tumour growth and survival. It is also possible, however, that only modest changes in PKB activity are required for the antiapoptotic effect this kinase provides.

DISCUSSION

In the present study, we report for the first time that the expression of ILK is significantly increased in sporadic colon cancer and metastatic deposits in regional lymph nodes, thus substantiating our original findings in colon polyposis. We also show that increased immunoprecipitated ILK MBP phosphotransferase activity was evident in 63% of the cases. In addition to this, we demonstrate that elevated ILK expression and increased MBP phosphotransferase activity coincide with effects on downstream targets of ILK signalling such as GSK3β phosphorylation. Based upon the data presented here and in keeping with the ‘just right’ model for colorectal carcinogenesis, which states that specific APC genotypes are selected during tumour formation on the basis of the specific level of residual β-catenin downregulating activity that is retained, additional signals are likely required for the development of human colon cancer (Albuquerque et al., 2002). Perhaps ILK, via its effects on Wnt signalling, acts in concert with the loss of APC function to facilitate disease progression. It is worth adding that a fine balance must exist between those signals that influence Wnt signalling in a positive vs a negative manner since excessive accumulation of β-catenin has been reported to coincide with the induction of apoptosis (Kim et al., 2000).

One of the important observations made in these studies was the identification that GSK3β is phosphorylated at Ser-9, which is indicative of its inhibition. This could be of primary importance as it suggests that (pre)malignant cells retain the ability to modulate signalling pathways which ultimately regulate the subcellular distribution of β-catenin and that this regulation is probably a consequence of the ‘second hit’ in the wild-type APC allele (Albuquerque et al., 2002). This is in agreement with the ‘just right’ model. It is possible that ILK-mediated inhibition of GSK3β could destabilise the formation of the ‘destruction complex’ since phosphorylation of axin and APC by GSK3β is said to favour the formation of the complex (reviewed in Fodde et al., 2001). In addition to this, inhibition of GSK3β by ILK could tip the scale in favour of enhanced growth. Overexpression of ILK has been shown to promote anchorage-independent cell cycle progression, which is likely mediated by the upregulation of Tcf4-dependent gene transcription as well as by the inhibition of GSK3β. Interestingly, GSK3β is known to phosphorylate cyclin D1; phosphorylation is essential for degradation of the latter by the ubiquitin–proteasomal complex (Diehl et al., 1998). It is well established that the levels of cyclin D1 are increased in this disease (Krišt et al., 2000; Utsunomiya et al., 2001). In addition to the putative effects of GSK3β on growth, inhibition of this kinase could favour cell survival. A number of studies have shown that GSK3β can modulate apoptosis (Frame and Cohen, 2001; King et al., 2001).

As outlined above, 63% of the cases in which the biochemical activity of ILK was assessed displayed changes in the MBP phosphotransferase activity, whereas all of the lesions evaluated using immunohistochemistry demonstrated significant changes in ILK expression. This discrepancy between the expression and activity of ILK could be attributable to various factors involved in tissue sampling, such as the extent of tumour vascularisation and time of harvesting (normally less than 2 h) or even quite possibly because of differences in the proposed etiological pathways, that is, MIN vs CIN. However, in conjunction with our previous findings in FAP, it is plausible to assume that overexpression of and increased activity of ILK could be an important event not only in the initiation of colorectal carcinogenesis but as well in the progression of the tumour towards invasion (Troussard et al., 2000). The transition in mammary epithelium (Somasiri et al., 2001) has been reported to result in the downregulation of E-cadherin (Novak and Dedhar, 1999), whereas administration of a selective inhibitor of ILK has been reported to result in the induction of E-cadherin expression (Tan et al., 2001). Certainly, ILK has been reported to promote epithelial to mesenchymal transition in mammary epithelium (Somasiri et al., 2001). In order to specifically address the role of ILK activity at the invasive front, the development of specific antibodies capable of detecting the activated form of ILK would be required. We were unable to demonstrate any significant changes using an antibody specific for the activated form of PKB.

In summary, we suggest that dysregulation of ILK signalling is an important early event in the genesis of human colon cancer. Furthermore, with the recent surge in the development of specific inhibitors to protein kinases, we propose that an inhibitor to ILK should be explored as a possible novel strategy for either the treatment and/or prevention of this disease. An inhibitor to this protein kinase could prove to be as efficacious as the tyrosine kinase inhibitor STI-571, which is used in the treatment of chronic myeloid leukaemia (Thiesing et al., 2000; Joensuu et al., 2001). Moreover, administration of an ILK inhibitor in combination with other chemotherapeutic drugs might prove to be as useful as the EKI-569 (a reversible inhibitor of the EGF receptor tyrosine kinase) sulindac combination (Torrance et al., 2000). Currently, we are evaluating whether changes in ILK expression/activity occur predominantly in CIN vs MIN lesions. As well, we are attempting to unravel the mechanism by which ILK is
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