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Receptor protein tyrosine kinase EphB4 is up-regulated in colon cancer

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Abstract

Background: We have used commercially available cDNA arrays to identify *EphB4* as a gene that is up-regulated in colon cancer tissue when compared with matched normal tissue from the same patient.

Results: Quantitative RT-PCR analysis of the expression of the *EphB4* gene has shown that its expression is increased in 82% of tumour samples when compared with the matched normal tissue from the same patient. Using immunohistochemistry and Western analysis techniques with an EphB4-specific antibody, we also show that this receptor is expressed in the epithelial cells of the tumour tissue and either not at all, or in only low levels, in the normal tissue.

Conclusion: The results presented here supports the emerging idea that Eph receptors play a role in tumour formation and suggests that further elucidation of this signalling pathway may identify useful targets for cancer treatment therapies.

Background

Colorectal cancer (CRC), arising from epithelial cells lining the large intestine, is the most commonly occurring internal cancer among Australians. Of all forms of cancer, it has the second highest mortality rate for both men and women, after lung cancer and breast cancer, respectively. Recently in Australia, the screening of all individuals over the age of 50 years for colon cancer has been proposed but although several genes may have good prognostic potential, a reliable genetic test for this disease has not yet been developed [1–3]. Further to this problem, up to 30% of patients undergoing supposed curative resection for early stage disease will relapse and die within 5 years, suggesting that metastatic spread is already underway in these patients.

In an effort to identify new markers for colon cancer, we have screened cDNA arrays to identify genes that show marked up-regulation in colon tumour cells when compared with matched normal mucosa. *EphB4*, a member of the largest known family of receptor protein tyrosine kinases, was identified as a gene that showed consistent up-

regulation in the patient tumour samples, when compared with the normal tissue, by array analysis. Eph receptors are involved in many cellular processes including neural development, angiogenesis and vascular network assembly [4–9]. They are large multi-domain membrane-anchored proteins consisting of six different regions [10] including a globular domain and a SAM domain, for which crystal structures have been determined [11,12]. As a result of Eph-ephrin binding, the receptor autophosphorylates allowing interaction with a variety of different proteins that regulate cytoskeletal organisation and cell motility [13,14]. The role of EphB4 and other Eph receptor family members in cancer has not yet been defined, although up-regulation of the expression of several of these has been demonstrated in tumours and cells lines from gastric tissues, prostate, breast, endometrium, and lung [15–23]. Here we present the results of experiments designed to further investigate the expression of this gene and to clarify its biological relevance to the progression of human colorectal adenocarcinomas. These results suggest that pathways involving Eph-ephrin signalling may be important in the progression of colon cancer and that therapies that target this receptor may find application in anti-cancer treatments.

Results

cDNA array analysis

Clontech Atlas expression arrays were differentially screened with cDNA probes made using RNA extracted from 2 pairs of matched tumour and normal mucosa samples (2 different patients) to identify genes that were up-regulated in tumour tissue. Images were visualised using a phosphorimager. Comparison of two different tumour/normal pairs identified EphB4 as consistently up-regulated in the tumour samples when compared to the matched normal tissue (ranked as the gene with the greatest difference in one patient and third greatest difference in the other using a proprietary algorithm developed by us) and it was therefore chosen for further analysis.

Identification of a pseudogene sequence corresponding to EphB4 gene

Primers EphB4F1 and EphB4R1 were designed to amplify a 466 bp product corresponding to bases 1377 – 1843 of the EphB4 cDNA sequence. Comparison with the EphB4 genomic sequence (Accession AY056047), shows that these primers span 4 introns and approximately 6 kb, allowing distinction of the cDNA and genomic products (a genomic product will not be synthesised under the conditions used in the RT-PCR analysis). However, when these primers were used in a PCR reaction using genomic DNA from six different patients as the template, bands of a similar size to the cDNA product were amplified (result not shown). The product from one of these patients was sequenced fully in both directions and it did not differ significantly from the EphB4 cDNA sequence. Because this suggested the presence of a pseudogene, further primers (EphB4F3 and EphB4R3) corresponding to bases 1819 – 3005 of the EphB4 cDNA sequence were designed to amplify a larger cDNA product of 1187 bp. As these did not amplify a product from the genomic template (primers span >10 kb), they were used for the RT-PCR analysis described later.

Expression of EphB4 in colon cancer cell lines

The expression of EphB4 was assessed for its suitability as a tumour specific marker using RT-PCR. RNA was extracted from ten colon cancer cell lines, reverse transcribed into cDNA then PCR-amplified using primers specific for the different genes. A primer set specific to CK19, a well characterised marker of colon epithelial cells [25–27], was used as a control for confirming integrity of the RT reaction. Transcripts corresponding to EphB4 and CK19 were amplified from all of the 10 cell lines (Figure 1).

Expression of EphB4 in primary colon tumours

Total RNA was extracted from 62 tumours and matched normal mucosal tissue taken from 60 patients (1 patient had three separate tumours) undergoing resection for primary colon cancer. Total RNA from liver metastases consistent with colorectal primary tumours, and normal liver samples from five of the CRC patients was also isolated. RNA was reverse transcribed and PCR amplified to compare levels of expression of each of the selected genes in the tumour and the matched normal mucosa. Semi-quantitative results were obtained by amplifying CK19 transcripts at the same number of cycles (Figure 2). As the same amount of RNA was transcribed for each sample at the same time, variation in the amount of CK19 amplified product in the cDNA samples may reflect normal variation in expression of these genes in the tumour and matched normal tissue. The amplification of this marker also demonstrates the integrity of the cDNA used for the EphB4 analysis. EphB4 showed increased expression in 30/38 (79%) of tumours analysed (as compared with the normal mucosa). As expected, from analysis of data in the EST databases, we did find that the EphB4 gene was expressed in normal liver, but it was also expressed to a similar level in the liver metastasis from patient 5 (consistent with colon origin). Analysis of other liver metastases will determine whether the apparent decrease in expression in the secondary tumour deposit when compared to the primary tumour is a consistent observation.

To quantify the expression of EphB4 in the tumour and normal samples for direct comparison of gene expression we used relative RT-PCR (Ambion). The amount of EphB4 transcript was quantified by concurrently amplifying a 489 bp fragment of the 18S ribosomal unit as an internal control in a multiplexed reaction (Figure 3). Using a gel

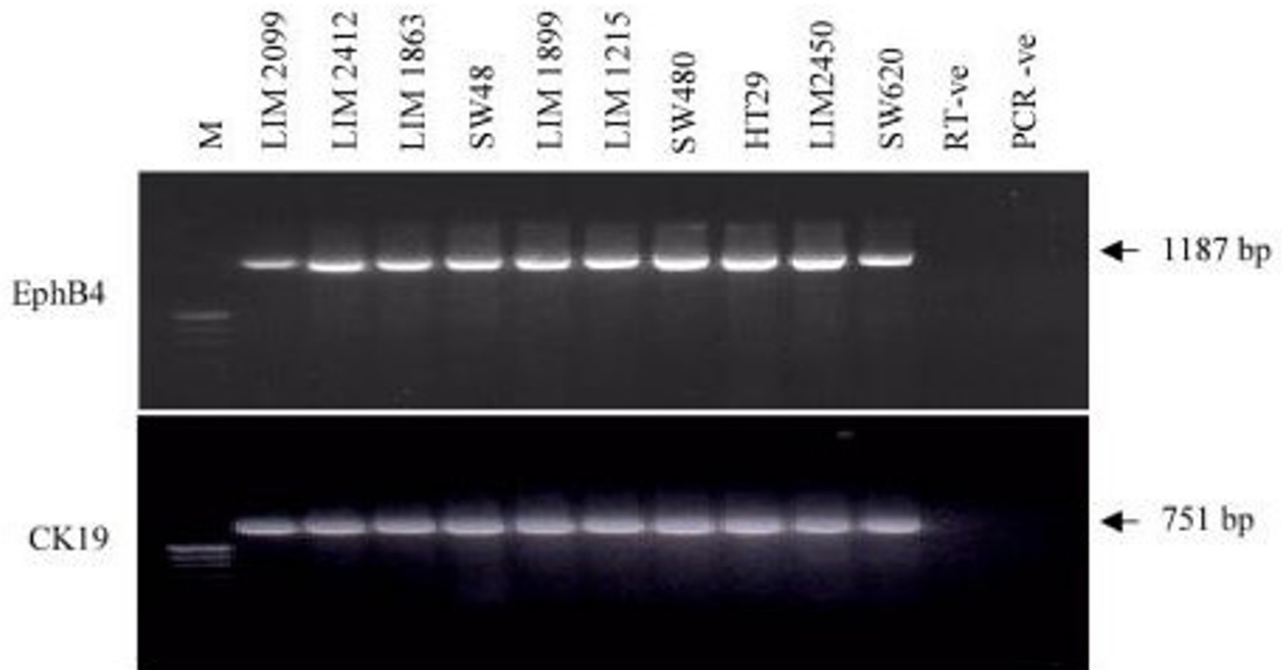

Figure 1
Expression of EphB4 and CK19 in ten colon cancer cell lines -LIM2099, LIM2412, LIM1863, SW48, LIM1899, LIM1215, SW480, HT29, LIM2405 and SW620. Sizes of the amplified products are indicated on the right. RT-ve – RT negative control, PCR -ve – PCR negative control, M – pUC19 *Hpa*II marker.

documentation system, the expression of the EphB4 gene was calculated relative to the level of 18S rRNA amplified from the same sample in the same PCR tube, and expressed as a ratio. This allowed direct comparison of the tumour and normal tissue paired samples. Quantification of both the 18S product and the EphB4 product showed that EphB4 was up-regulated greater than 1.5 times in 51/62 (82%) of tumour samples when compared with the normal mucosa and greater that 2 times in 39/62 (63%). An ANOVA test of the results of three identical experiments using seven paired tumour/normal samples showed there was no statistical difference between experiments. A paired, matched Student's T-test showed that the difference between the levels of EphB4 expression in the tumour tissues and the levels of EphB4 expression in the normal tissues was significant ($P < 0.0001$).

Western analysis confirms that EphB4 protein is increased in colon tumours and low in matched normal mucosa

To determine if the increased expression of the *EphB4* gene corresponded to an increase in the EphB4 protein we performed western analysis of colon and matched normal tissues using an EphB4-specific polyclonal antibody. The predicted size of wildtype EphB4 protein has been estimated by Bennet et al., (1994) to be 120 kD and in our experiment a band of this size was present in protein extracts from the tumour samples but low or absent in extracts from the normal mucosa (Figure 4) [28]. This confirms that the increased expression in the tumour tissues does correspond to an increase amount of the protein itself. Additional signals corresponding to proteins of lower molecular weight were also visible specifically in the tumour samples. It is unknown at this time whether these are EphB4-specific cleavage products or not and this will be the subject of future investigation. Further samples will be need to be tested to determine if there is any association between the stage of tumour development and amount of protein present.

Immunohistochemical analysis of EphB4 expression in colon tumour and matched normal mucosa

To localise the expression of EphB4 in normal and tumour tissue, we performed immunohistochemistry on 6 matched samples using an EphB4 specific antibody. The results for three patient sample sets are presented in Figure 5. The epithelial layer in the normal tissue and a well differentiated and a moderately-well differentiated tumour can be clearly distinguished by the single row of regular nuclei (stained blue with Harris Haematoxylin) lining the crypts. The most intense staining was localised to the tu-

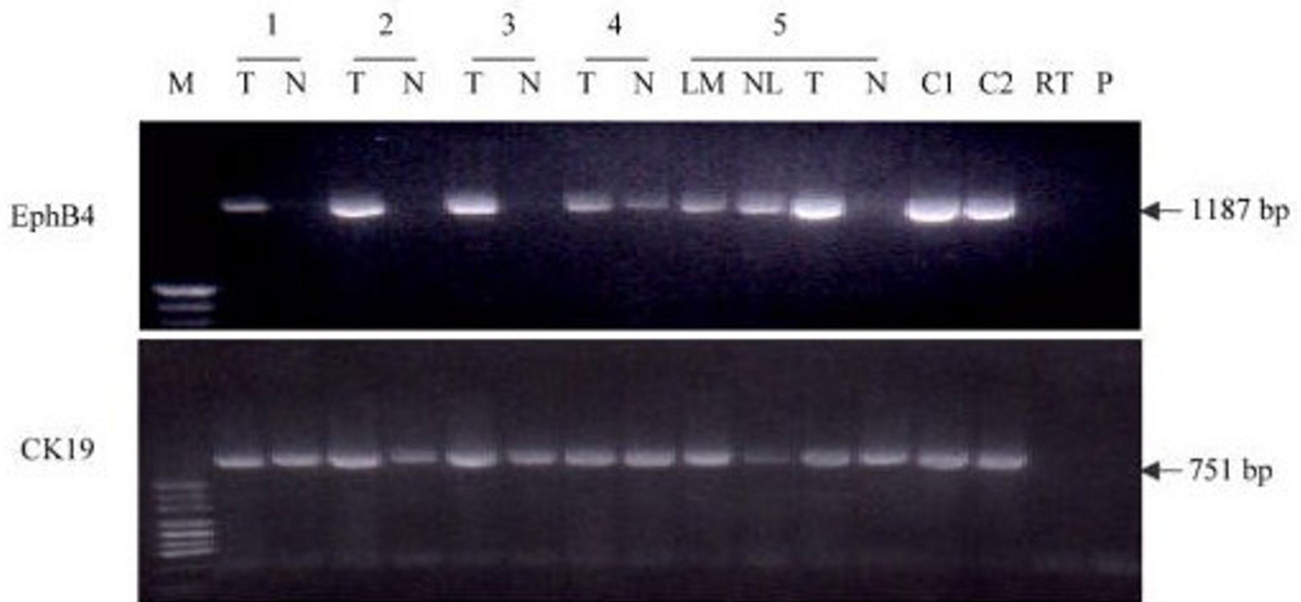

Figure 2
Semiquantitative analysis of EphB4 gene expression in five tumour (T) and normal (N) pairs, one of which includes a liver metastasis consistent with the colorectal primary from this patient (LM) and a normal liver sample (NL), by comparison with CK19 expression. Sizes of the amplified products are indicated on the right. C1 – colon cancer cell line LIM2405, C2 – colon cancer cell line SW480, RT- RT negative control, P – PCR negative control, M -pUC19 *Hpa*II marker.

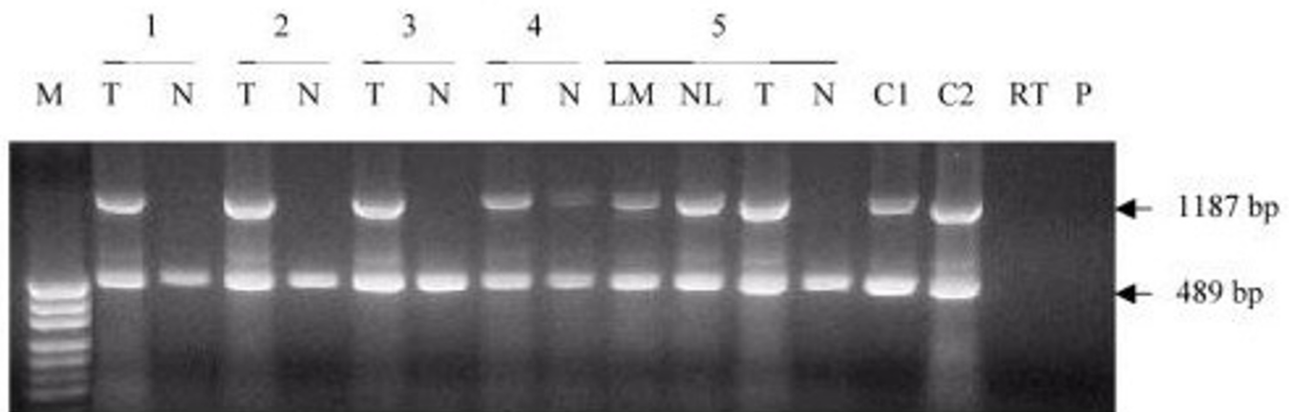

Figure 3
Relative RT-PCR comparing expression of EphB4 (1187 bp) and internal 18S rRNA (489 bp) in the same five tumour (T)/ normal (N) pairs shown in Fig 4. C1 – colon cancer cell line LIM2405, C2 – colon cancer cell line SW480, RT- RT negative control, P – PCR negative control, M – pUC19 *Hpa*II marker.

mour epithelial cells as shown by the brown staining. Both the absorptive, surface epithelial cells and crypt mucus-secreting cells of the tumour tissue showed strong staining at the cell membranes with comparable intensity at both the apical and basal surfaces. The poorly differentiated tumour appears to be a mass of EphB4-expressing cells. There was no obvious staining of endothelial cells of blood vessels, confirmed by immunohistochemical analysis of human umbilical vein endothelial cells grown on slides (data not shown) and only weak diffuse staining in

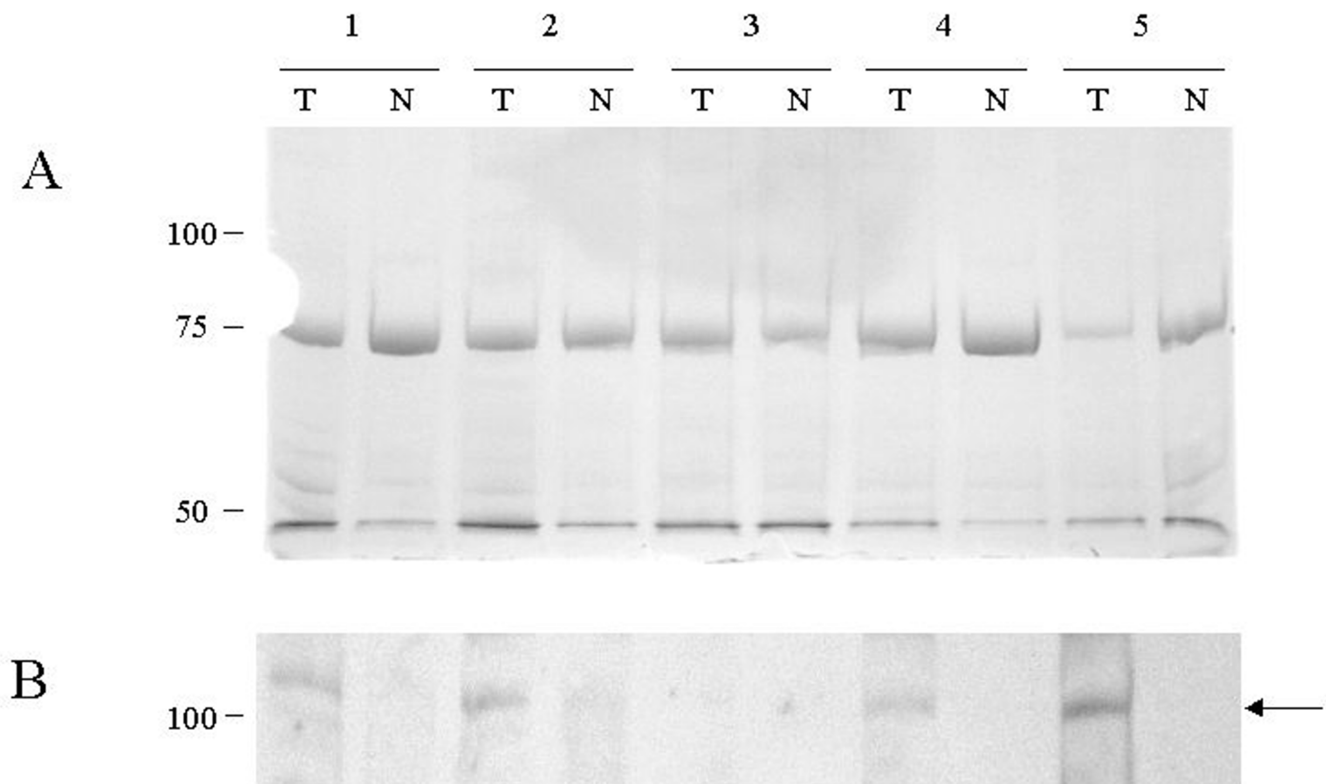

Figure 4
(A) Coomassie stained duplicate gel for loading comparison. (B) Western analysis of EphB4 protein expression in colon tumour (T) and matched normal mucosa (N) from five different patients. The size markers in kDa are shown on the left. The arrow indicates the normal EphB4 protein.

the normal tissue. There was no staining with the secondary antibody alone (results not shown).

Discussion

Currently, tumour-specific gene markers for colon cancer are not available and further characterisation of gene expression in tumour cells is required. The development of cDNA array technology has enabled the expression analysis of a large number of genes using differential screening techniques. We have used cDNA arrays (Clontech) to analyse the expression pattern of 588 genes, chosen for their defined roles in processes such as oncogenesis, proliferation and apoptosis. A combination of data from two different patient samples identified EphB4 as a potential candidate marker and relative RT-PCR analysis and immunohistochemistry has been used to demonstrate that up-regulation of expression of this gene in tumour epithelial cells is a common feature of colon tumours.

Up-regulation of the expression of several members of the Eph family of receptor tyrosine kinases has been associated with cancers from breast, lung and gastric tissues which implies an important function for these proteins in the development and/or function of the tumour cells [15–23] Protein tyrosine kinases collectively make up the largest family of oncogenes. Since they function in signal pathways that control cell shape, proliferation, differentiation and migration, there would seem to be many potential ways in which their dysregulation could promote tumorigenesis (reviewed in [29] and [30]).

It is possible that Eph receptors and ephrins have parallel or co-operative roles in cell adhesion systems and in restricting cell intermingling during development. Studies of EphR signalling during embryonic development show that these receptor molecules are involved in processes which alter the cell cytoskeleton and may regulate the function of cell adhesion molecules causing de-adhesion of cells at boundaries [6]. Alternatively or in addition, activation may trigger cell repulsion responses involving the localised collapse of the actin cytoskeleton. Zisch et al (2000) have used a panel of EphB2 mutants to show that receptor kinase activity is required for changes in cell-matrix and cell-cell adhesion and cytoskeletal organisation in transiently transfected cells [31]. Eph-ephrin signalling may also be involved in the demarcation of arterial and

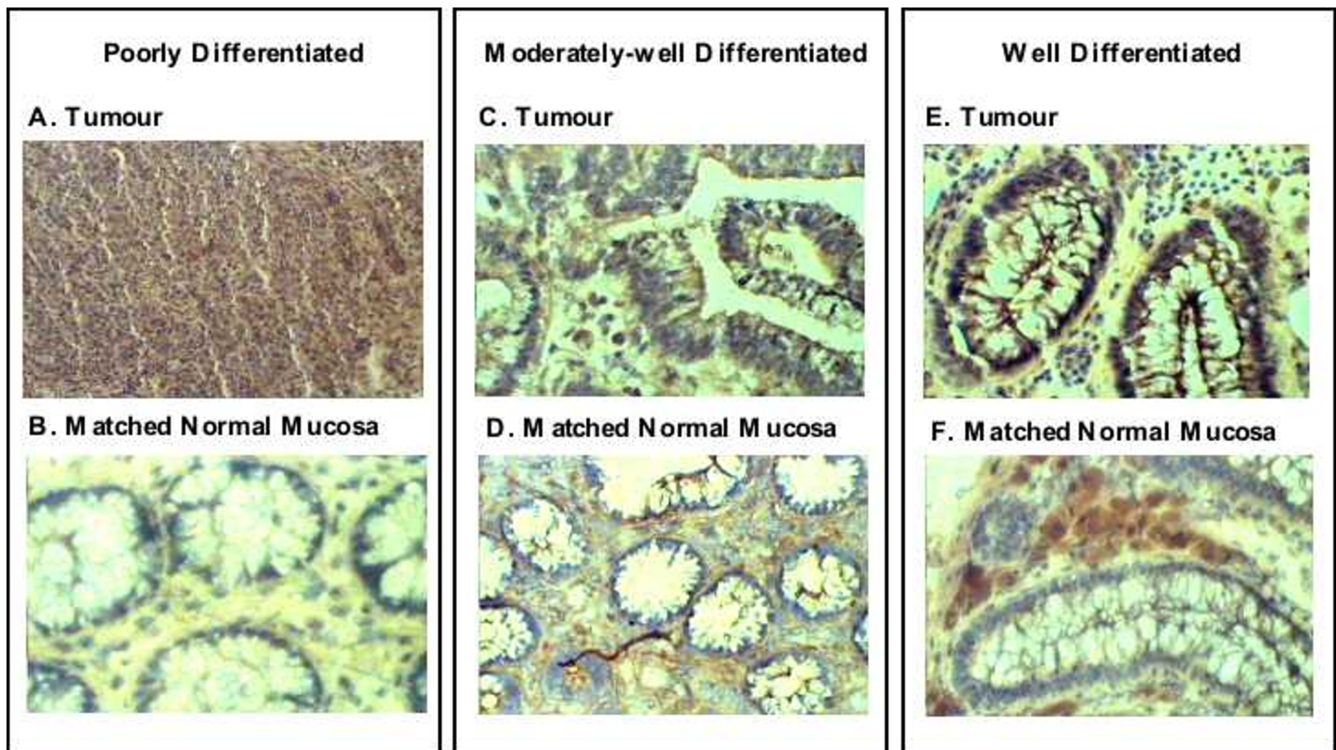

Figure 5
Immunohistochemical localisation of EphB4 expression in three different colon cancers and matched normal mucosa. The brown stain from the biotinylated secondary antibody indicates the EphB4 protein. Nuclei are stained with Harris haematoxylin and appear blue. High power (100X) magnification images of three different adenocarcinomas (well differentiated, moderately well differentiated and poorly differentiated) and their matched normal mucosa are shown. Strong staining of the tumour tissue and very weak, diffuse staining of normal tissue was evident for each sample set. There was no cross-reactivity with the secondary antibody alone (result not shown).

venous vessels and the recruitment of endothelial cells to the formation of new vasculature [8,32]. Adams et al (2001) have shown that embryos expressing the EphB4 ligand ephrnB2 lacking the cytoplasmic domain have defects in vasculogenesis and angiogenesis and this results in midgestation lethality similar to ephrinB2 null mutants [33]. EphrinB2 is a promiscuous ligand that binds to a number of EphB receptors and may regulate many different functions. This may explain why these mutations are lethal. However these results in combination imply that Eph receptor signalling may regulate changes in cell-matrix and cell-cell adhesion and cytoskeletal organisation while ephrin ligand signalling is important for vasculogenesis and angiogenesis.

Of particular reference to a proposed function in cancer development is the demonstration that Eph-ephrin signalling can prevent the formation of gap junctions between neighbouring cells, as do the products of various oncogenes including src, neu, raf, fps and ras [34,35]. The loss of gap junctional communication correlates with progression to a malignant phenotype for many cancer cell types [35].

In the Eph-ephrin signalling system, Stein et al. (1998) have shown that the density of ephrin plays an important part in interpreting different signals [36]. Tetramers of ephrins signal through EphRs to induce cell adhesion in a heterologous system and in primary cultures but the dimeric form is ineffective in this function even though both forms are equally effective in eliciting EphB1 autophosphorylation. If EphB4 is overexpressed in colon tumour cells, ephrins may no longer be able to form tetramers and may therefore not induce cell adhesion, which would then assist in the spread of cancer cells. However, the fact that the dimeric form can also induce receptor autophosphorylation suggests that this may also be an important regulatory system in other, as yet unknown, ways.

Tumour cells need to acquire a vascular network if they are to grow beyond a few millimetres in diameter. Because EphB4 has been characterised as a key player in the path-

way that distinguishes arteries and veins [8,37], it was possible that the up-regulation of expression of EphB4 was actually in stromal cells such as the endothelial cells recruited by the tumour cells for this network. We used immunohistochemical techniques and an EphB4-specific antibody to identify which cells within the tumour tissue were expressing the EphB4 protein. Our results have shown that the up-regulation of the EphB4 mRNA corresponds to an increase in the amount of EphB4 protein produced within the tumour tissue for all six patients analysed and that this expression is a localised in the tumour epithelial cells with only weak, diffuse staining in the normal mucosa and stromal cells.

Conclusions

Increased expression of the EphB4 gene suggests that EphB4 signalling may play an intrinsic role in the development of a tumour phenotype in colon cancer and raises questions concerning this potential role. Further experiments, including targeted disruption of the EphB4 gene in colon cancer cells and testing the effect of this on the ability of the tumour cells to grow in vitro and in vivo, may provide useful information for developing strategies for treatment of this disease. Furthermore, the identification of factors that activate the transcription of EphB4 in colon cancer cells may identify signalling mechanisms that are specific to tumour cells. The elucidation of these early events in the signal transduction pathways that lead to development or progression of the tumour cell phenotype may assist in the development of novel detection or prevention strategies.

Materials and Methods

Tissue Samples

Tumour and normal tissue were collected at the time of resection from 60 patients (35 male, 25 female), median age 69 years (range 43–91 years) with colorectal cancer at The Queen Elizabeth Hospital, Adelaide, Australia, and snap frozen in liquid nitrogen. All tumour tissue was histologically confirmed as adenocarcinoma, and staged using the ACPS classification system. Liver metastases consistent with colorectal primaries were also collected from five of these patients after hemi-hepatectomy. Informed consent was obtained in all cases and ethics approval was obtained from The Queen Elizabeth Hospital Ethics Committee.

Cell culture

Colon cancer cell lines SW480, SW620, SW48, LIM1215, LIM2405, HT29, LIM1863, LIM2412 and LIM2099 were cultured in HEPES-buffered RPMI 1640 medium (Life Technologies, Grand Island, N.Y.), pH 7.4. The medium was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 160 μg/ml L-glutamine and 10% heat-inactivated foetal bovine serum (JRH Biosciences, Lenexa, KS) in 75 cm^2 vented tissue culture flasks at 37°C in a 5% CO_2 environment. Cells were collected at >90% confluency by trypsin digestion and centrifugation for 5 min at 1000 rpm, resuspended in phosphate buffered saline (PBS) and counted using a haemocytometer. The LIM cell lines were kindly provided by Dr R. Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia); the remainder were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

RNA and protein extraction from tissues and cell lines

RNA and protein were isolated sequentially from tissue samples and cell lines using Trizol™ solution (Sigma, St. Louis, MO). Growth medium was aspirated from the flask of growing cells (>90% confluency) and the cells washed with phosphate-buffered saline (PBS) before being directly lysed using 1 ml of the Trizol™ solution. After a five min incubation with gentle rocking, the cell lysate was removed to a 2 ml eppendorf tube. Tissue samples (0.5 – 1.0 g) were ground in liquid nitrogen using a mortar and pestle, then transferred to an eppendorf tube containing 1 ml Trizol™ solution. The RNA and protein fractions were extracted from each of these samples using the manufacturer's recommendations. Protein concentrations were determined using the *DC* Protein Assay Kit from Biorad (Sydney, NSW, Australia) following the manufacturer's protocol and using bovine serum albumin diluted from 0.2 mg/ml to 1.5 mg/ml to determine the standard curve.

cDNA array analysis

Two duplicate (from the same manufacturing batch) Atlas Cancer cDNA arrays (Clontech, Palo Alto, CA) were differentially screened with cDNA probes made using mRNA extracted from tumour and matched normal tissue from two different patients and the supplied control mRNA. Total RNA (25 μg) was DNase treated (Life Technologies, Bethesda, MD) then re-extracted using Trizol™ solution. mRNA was prepared from these samples using the Oligotex mRNA purification kit (Qiagen, Germany) following the manufacturer's protocol. cDNA probes were synthesised using the method supplied with the cDNA array filters. These ^{32}P-labelled probes were hybridised to different arrays in 15 ml ExpressHyb™ solution (Clontech) overnight at 68°C, then washed at high stringency in the recommended wash solutions. Blots were exposed to phosphorimager plates overnight, then visualised using a Fuji-BAS phosphorimager. The blots were re-hybridised with a new probe after removal of the previous probe by incubation in boiling 0.1% SDS for 10 min and confirmation by phosphorimaging that there was no residual signal retained.

Semi-quantitative and Relative RT-PCR

Total RNA (2 μg) was reverse transcribed at 37°C using 3 μl $pD(N)_6$ primers (Life Technologies), 200 μM each de-

oxyribonucleoside triphosphate (dNTP) (Pharmacia, Uppsala, Sweden) and 200 U MMLV reverse transcriptase (Life Technologies) in a reaction volume of 30 μl. Primers specific to either EphB4 (EphB4F1 5'- GAG AGG TAC CTC CTG CAG TGT C and EphB4R1 5'- CCA TGT CCG ATG AGA TAC TGT CCG-3' or EphB4 F2 5'-CGG ACA GTA TCT CAT CGG ACA TG-3' and EphB4 R2 5'-GCT TGG CCT GGG ACT TCA TGT G-3') or CK19 (CK19 F 5'-GAC TAC AGC CAC TAC TAC ACG ACC and CK19 R 5'-AGC CGC GAC TTG ATG TCC ATG AGC C), were used in a PCR reaction carried out in a Perkin Elmer thermocycler with the following conditions: 1.5 mM $MgCl_2$, 200 μM each dNTP, 50 ng of each primer, and 0.5 units of Tth polymerase in 1 × PCR buffer (10 × PCR buffer is 670 mM Tris-HCl pH 8.8, 166 mM $[NH_4]_2SO_4$, 4.5% Triton X-100, 2 mg/mL Gelatin) (Biotech International, Perth, Australia). Cycling conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C (EphB4 set 1) or 63°C (EphB4 set 2) or 68°C (CK19) for 30 sec, and 72°C for 30 sec, with a final extension of 72°C for 7 min. The QuantumRNA™ 18S Internal Standards kit from Ambion (Austin, TX) was used to calculate the relative abundance of transcripts corresponding to EphB4. One microlitre of a 3:7 ratio of 18S primer to 18S competimer (product of 489 bp) was added to each reaction tube and the annealing temperature increased to 62°C as recommended in the Ambion protocol. PCR products were separated by electrophoresis through 1.5% agarose gels and the results analysed using the Kodak Digital Science ID 2.0.2 program (Eastman Kodak Company, New Haven, CT, USA). The level of EphB4 expression in each sample was calculated relative to the amount of 18S product within that sample (expressed as a ratio). The relative expression of tumour compared to normal was compared using the GraphPad Instat™ program (http://www.graphpad.com/instat3/instat.htm).

Western Analysis

Protein samples (20 μg) extracted from tumour and matched normal tissue were analyzed in duplicate using 6% SDS-PAGE gels [24]. One of these gels was stained with coomassie blue to visualise the proteins and compare loading of samples and the second was used for transfer to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA). EphB4 antigens were detected using a 1:1000 dilution of the rabbit polyclonal antibody EphB4 (H-200) (Santa Cruz Biotechnology, Santa Cruz, CA) raised to human EphB4. After an overnight incubation at 4°C, the primary antibody was detected using the Lumi-Light^{PLUS} Western Blotting kit (Roche Biochemicals, Mannhein, Germany) following the manufacturer's recommendation, and between 10s and 30s exposure to Hyperfilm™ ECL™ (Amersham Pharmacia Biotech, UK).

Immunohistochemistry

Sections (8 μm) of fresh frozen tissue were mounted on poly-L-lysine coated slides, fixed in acetone, air dried, then frozen until required at -80°C. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min at room temperature. Nonspecific binding sites were blocked with 3% normal goat serum in PBS for 20 min at room temperature and the Vector Laboratories (Burlingame, CA) Avidin/Biotin blocking kit following the manufacturer's instructions. The sections were then incubated overnight at 4°C with a 1:200 dilution of the EphB4-specific antibody used for the western analysis. After rinsing with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min at room temperature followed by washing with PBS. Immunoreactivity was detected with the avidin-biotin system (Vector Laboratories) using 18.5 mM 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as a chromogen for 2 min. The sections were then counterstained using Harris haematoxylin, dehydrated, cleared using SUB-X clearing solution (Surgipath Medical Industries, Inc. Richmond, IL) and mounted using SUB-X mounting medium (Surgipath).

Competing interests

None declared

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