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Complex expression patterns of Eph receptor tyrosine kinases and their ephrin ligands in colorectal carcinogenesis

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ABSTRACT

Aberrant expression of Eph and ephrin proteins in human cancers is extensively documented. However, data are frequently limited to one gene and therefore incomplete and in some instances conflicting. We analysed expression of all Eph and ephrin genes in colorectal cancer (CRC) cell lines and 153 clinical specimens, providing for the first time a comprehensive analysis of this system in CRC. Eph/ephrin mRNA expression was assessed by quantitative real-time PCR and correlated with protein expression (flow cytometry, Western blotting and immunocytochemistry). These data show that EphA1, EphA2, EphB2 and EphB4 were significantly over expressed in CRC. In all cases, at least one Eph gene was found in normal colon (EphA1, EphA2, EphB2, EphB4), where expression was observed at high levels in most CRCs. However, other Eph gene expression was lost in individual CRCs compared to the corresponding normal, EphA7 being a striking example. Loss of expression was more common in advanced disease and thus correlated with poor survival. This is consistent with the redundant functionality of Eph receptors, such that expression of a single Eph gene is sufficient for effector function. Overall, the data suggest a progressive loss of expression of individual Eph genes suggesting that individual CRCs need to be phenotyped to determine which Eph genes are highly expressed. Targeted therapies could then be selected from a group of specific antibodies, such as those developed for EphA1.

1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related mortality in the United States of America (USA), reflecting the ineffectiveness of therapy in tumours that have spread beyond the bowel wall. The need for new therapies has focused attention on molecular mechanisms of CRC initiation and progression in a search for molecular targeted therapeutic approaches. Amongst the genes implicated in later stages of CRCs are members of the receptor tyrosine kinase (RTK) family including the largest RTK subfamily, the Eph RTKs.[1]

The sixteen Eph RTKs are divided into two groups based on structural features and binding affinities to ephrin ligands.[2] The 10 EphA RTKs preferentially bind the six glycophosphatidyl inositol (GPI) linked A ephrins, and the six EphB RTKs

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preferentially bind three type-B transmembrane ephrins.[3] Eph and ephrin proteins on juxtaposed cells interact to initiate both Eph-mediated 'forward signalling' and ephrin-mediated 'reverse signalling'.[1] In both cases the principal targets of these signals are the cytoskeleton and cell adhesive mechanisms thus regulating cell shape, cell position and motility.

Ephs and ephrins are expressed at high levels in many human cancers including gastrointestinal malignancies.[4–6] This has led to the notion that dysregulated function contributes to an invasive and metastatic tumour phenotype. A number of studies point to the involvement of Eph genes in CRC. EphB2 and EphB3 regulate cell movement in the normal gut and have also been shown to have a role in tumours in $Apc^{Min/+}$ mouse model of CRC.[7] Interestingly, in late stage lesions some tumours lost EphB expression leading to heterogeneity of expression which is in keeping with data on human CRC where EphB2 up-regulation is observed in only a subset of CRC.[8] Persistent high expression of both EphB2 and EphB4 have been associated with prolonged survival,[8,9] suggesting that loss of expression parallels acquisition of a more aggressive tumour phenotype.

There is less data on EphA/ephrin A expression in CRC. Significant expression of human EphA1 and EphA2 have been observed in a number of epithelial tumours including CRC.[10,11] EphA2 expression was further shown to be correlated with metastatic behaviour.[11] Ephrin A1 is the high affinity ligand for both EphA1 and EphA2 suggesting that EphA1/EphA2/ephrin A1 may form a partially redundant signalling system in epithelial tissues. A study of EphA2 and ephrin A1 in the $CaCo_2$ colon cancer cell line suggests that this interaction may be of importance in colon epithelial structure and function.[11] Interestingly, ephrin A1 down regulation in HT29 cells seems to inhibit *in vitro* invasiveness.[11] There is scant evidence for involvement of other EphA receptors in CRC. EphA7 expression has in fact been shown to be lost in most CRC apparently through epigenetic silencing.[12,13]

These studies imply a complex, poorly understood role for both EphA and EphB proteins in CRC but provide incomplete and somewhat conflicting data as to the role of this family. In this report we will show that a number of Eph proteins are expressed but in a variable manner leading to great heterogeneity. However, we will show that most if not all CRCs express high levels of at least one Eph receptor. Therapies targeting RTKs' are currently explored in breast cancer (Her2/Neu), lung cancer (EGF receptor), and cancer angiogenesis (VEGF receptor). Recent studies of the Eph family in angiogenesis show potential,[14] and understanding this heterogeneity is essential in correctly exploiting targeting therapies. This is discussed in light of our findings.

2. Materials and methods

2.1. Cell lines and clinical samples

Six CRC cell lines (LIM1215, $CaCo_2$, LISP-1, LoVo, HCT116, and HT29) were cultured in RPMI 1640 medium with 10% foetal bovine serum (FBS). $CaCo_2$ was cultured in Dulbecco's modified medium (Gibco, NY) with 20% FBS. Lines were maintained in a 5% CO_2 humidified incubator at 37 °C. A total of 153 colon specimens were assessed in this study, including colon tissue QPCR arrays HCRT101 and 501 (Origene technologies) consisting of 47 cDNA samples in each panel and a well characterised clinical cohort of 53 paired normal and CRC specimens. The QPCR arrays contained cDNA for five normal colon, six stage I, 18 stage II, 14 stage III and five stage IV CRC samples. The median age of the patients was 70.5 years (range 31–93 years) with a male to female ratio of 26:22. The 53 paired tumour and adjacent non-malignant samples were obtained from the Princess Alexandra Hospital tissue bank and the Royal Brisbane Hospital (Brisbane, Australia). Stages II and III were predominant in this cohort with only four stage I and three stage IV cancers. The median age of the patients was 72 years (range 29–84 years) with a male to female ratio of 28:25. All patient samples were obtained once specific informed consent procedures were approved by the Ethics Committees of the relevant institutions.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated using the QIAGEN RNeasy® Mini Kits (QIAGEN, Australia), according to the manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis. Prior to cDNA synthesis, samples were treated with RQ1 RNase-free DNase I (Promega, Australia), and first strand cDNA was synthesised by reverse transcription using Superscript III Reverse Transcriptase (Invitrogen, Australia) according to the manufacturer's instructions.

2.3. Relative quantitation by real-time PCR

Quantitative real-time PCR (QPCR) was carried out using Quantitect™ SYBR® Green PCR Master Mix (QIAGEN, Australia) following manufacturer's instructions. For tissue arrays, 1× Quantitect™ SYBR® Green PCR Master Mix was added to 13 μl of double distilled H_2O. Forward and reverse primers were added to a final concentration of 0.66 μM per reaction. Q-PCR was carried out in an ABI Prism 7900HT thermocycler (Applied Biosystems, USA).

For cell lines and CRC clinical samples, 5 μl of diluted cDNA was added to Quantitect™ SYBR® Green PCR Master Mix. Forward and reverse primers were added to a final concentration of 0.3 μM. Housekeeping genes β-actin, GAPDH, 18S rRNA and HMBS were all tested and showed similar results. β-actin was chosen in this instance as this showed a good coefficient of correlation for colorectal tissues in accord with other reports.[15] QPCR primer sequences are listed in Supplementary Table 2. All reactions were performed in duplicate to assess reproducibility. QPCR was carried out in a Corbett Research Rotor-Gene 3000™ (Corbett Research, Australia).

The PCR cycling conditions included activation for 15 min at 95 °C and 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Fluorescence data was recorded at the end of each 72 °C step. A DNA melt profile was run subsequently from 72 °C to 95 °C with a ramp of 1 °C/5 s. Amplification and detection conditions were identical when assaying gene expression by QPCR for both cell lines and tissue samples.

Copy number analysis relative to ß-actin was used to assess tissue arrays and the Pfaffl method of quantification was used for data analysis of the paired normal and tumour samples.[16] The relative expression ratio for Ephs and ephrins

was calculated using QPCR efficiencies and the crossing point deviation of an unknown sample versus an internal control (calibrator).

2.4. Development of EphA1 and EphA2 monoclonal antibodies

Mouse hybridomas were generated by fusion of immune splenocytes with SP/20 non-secreting myeloma cells according to established protocols.[17,18] For EphA1, mice from an EphA1 knockout strain generated in our lab[19] were hyperimmunised with mouse and human EphA1-Fc fusion protein.[20] After priming and two boosts with EphA1 Fc emulsified in Freund's adjuvant, sera were tested by immunofluorescence using an EphA1 transfected CHO cell line and by ELISA against EphA1-Fc. Animals showing high serum titres to EphA1 were used for hybridoma production. Clones were selected based on a positive ELISA for EphA1-Fc but not for control Fc protein.[21] For EphA2 hybridomas a similar method was employed except that EphA2 knockout mice[22] were immunised with human EphA2-Fc protein and clones screened on ELISA plates coated with EphA2-Fc or a control Fc.

2.5. Immunostaining

Immunocytochemical analysis of EphA1, EphA2, ephrin A1 and ephrin A5 was performed on CRC cell lines using the in-house anti-EphA1 mouse IgG1 monoclonal antibody (4B6), anti-EphA2 mouse IgG1 monoclonal antibody (1F7), anti-ephrin A1 rabbit IgG polyclonal antibody (Santa Cruz) and anti-ephrin A5 rabbit IgG polyclonal antibody (Santa Cruz), respectively. Briefly, formaldehyde fixed cells (2% formaldehyde for 10 min) were washed twice with PBS and incubated with primary antibodies for 20 min. Cells were washed twice with PBS-5% FCS and incubated with either Zenon™ Alexa Fluor® 594 Rabbit IgG or Zenon™ Alexa Fluor® 488 mouse IgG (Molecular Probes) for 15 min. The cover slips were mounted onto slides using the DAKO fluorescent mounting medium (Dako). Cells were scanned using a Leica TCS SP2 confocal microscope and Leica Confocal Software v2.7. The excitation source for Alexa 488 was a 20 mW Argon Laser @ 488 nm, whereas the excitation source for Alexa 594 was a 1.2 mW Green HeNe Laser @ 543 nm.

2.6. Flow cytometry

All washes and antibody binding steps used 5% FBS in PBS to reduce non-specific protein binding. 1×10^5 cells were washed three times and incubated with in-house anti-EphA1 mouse IgG1 monoclonal antibody (4B6) or anti-EphA2 mouse IgG1 monoclonal antibody (1F7) for 30 min at room temperature. The cells were washed twice and incubated for 15 min with an anti-rabbit FITC (Chemicon, Australia) at room temperature in the dark. After several washes the cell pellet was re-suspended in propidium iodide in PBS (20 μg/ml). Cell fluorescence (two-colour) was detected using a BD FACSCalibur™ and data acquired using CellQuest Pro v4.0.2. Subsequent analysis of fluorescence data was conducted using Summit® v3.1.

2.7. In vitro demethylation and histone deacetylase inhibition

To assess whether gene expression could be restored, cell lines displaying low or no expression (HT29 for EphA1, HCT116 for EphA2, HT29 for ephrin A1, LISP-1 for EphA3, EphA7 and ephrin A5) were treated with a methyltransferase inhibitor (5′-aza-2′-deoxycytidine) with or without a histone deacetylase (HDAC) inhibitor. A total of 4×10^5 cells were plated into petri-dishes and treated with freshly prepared 2 μM 5′-aza-2′-deoxycytidine (Sigma, Australia) with or without the HDAC inhibitor, suberic bishydroxamate (SBHA) at a final concentration of 30 μg/ml.[23] Cells were treated for periods of 24, 48 and 72 h with media and inhibitors replaced every 24 h. Cells were harvested at approximately 80% confluence after the completion of the treatments. QPCR was performed as described above.

2.8. Statistical analysis

Quantitative gene expression data from tissue arrays and paired normal and CRC samples was analysed using the non-parametric Wilcoxon Signed Ranks. All statistical analyses were performed using SPSS for Windows v15.0 and a p-value of <0.05 was considered statistically significant. Where appropriate, a Bonferroni adjustment was applied to p-values.

3. Results

3.1. Eph and ephrin expression in CRC cell lines

A survey of the expression of Eph genes, EphA1, A2, A3, A4, A5, A6, A7, A8, B1, B2, B3, B4, B6 and ephrin genes, A1, A2, A3, A4, A5, B1, B2, B3, was performed using Q-PCR in 6 CRC cell lines. Q-PCR analysis revealed a diversity of Eph expression (Supplementary Table 1). Notably, some cell lines showed high EphA expression and low to moderate EphB expression (LoVo, LIM1215, LISP, HT29) whilst others showed predominant EphB expression (LIM1863, $CaCo_2$).

EphA2 was the most highly expressed Eph gene in these CRC cell lines although the expression was quite variable. EphA1 was the only other EphA gene showing markedly elevated expression and, as with EphA2, a diversity of EphA1 mRNA expression was observed in CRC cell lines. There was little evidence of EphA3, EphA5, EphA6 and EphA8 expression amongst the cell lines. A modest level of EphA4 expression was observed in only one cell line, $CaCo_2$, and a low level of expression was seen in the HCT116 cell line. In contrast, ephrin A gene expression was relatively low to undetectable in these cell lines, although a moderate level of ephrin A5 expression was detected in LoVo.

In CRC cell lines, EphB2 and ephrin B1 were the highest expressing EphB and ephrin B genes, respectively. Significant levels (>five-fold) of EphB2, EphB3, B4 and ephrin B1 and B2 expression were seen in some cell lines whilst EphB1, B6 and ephrin B3 expression was negligible in these lines. Although there was an impression of a reciprocal relationship between expression of EphAs (EphA1 and EphA2) and EphBs (EphB2 and EphB4) this was not statistically significant and did not hold for all the lines (Fig. 1).

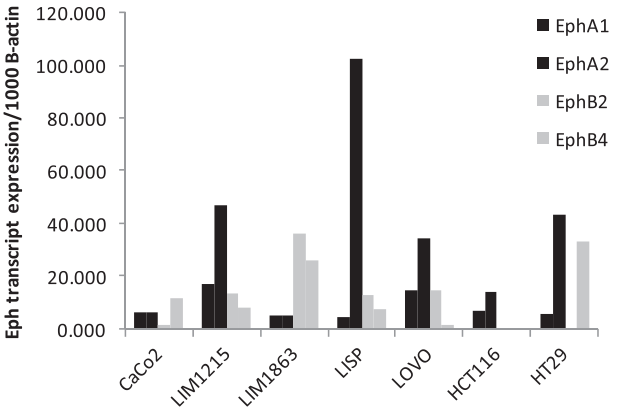

Fig. 1 – Reciprocal relationship between mRNA expression of EphAs (EphA1 and EphA2) and EphBs (EphB2 and EphB4) in CRC cell lines.

3.2. *Development of EphA1 and EphA2 monoclonal antibodies*

To further explore the levels of EphA1 and EphA2 on CRC cell lines we wished to confirm that levels of mRNA determined to be >five-fold would indeed correlate with significant cell membrane protein expression. To obtain useful antibody reagents we took advantage of knockout mouse strains available in our laboratory to prepare highly specific antibodies to these receptors. This strategy was devised after initial immunisation with highly purified EphA1 protein into normal mouse strains yielded only weakly reactive antibodies, presumably reflecting the highly conserved nature of these proteins in mammalian species. However, immunisations in EphA1 and EphA2 knockout mice and subsequent hybridoma production enabled us to develop high affinity anti-EphA1 (3E9, 4B6) and anti EphA2 (1F7) monoclonal antibodies.

The 4B6 anti-human EphA1 IgG1 and IF7 anti-human/mouse EphA2 IgG1 antibodies have been validated for ELISA, immunocytochemistry, flow cytometry (Fig. 2) and immunoprecipitation. In considering potential therapeutic applications, 3E9 anti-EphA1 IgG1 antibody has similar properties however, like 1F7, has the additional advantage of reacting equally with mouse and human EphA1, potentially enabling assessment of toxicity associated with binding to normal tissues in pre-clinical studies.[24] Furthermore, binding curves were generated for both 4B6 and 3E9 on a BIAcore optical biosensor for these antibodies to ensure that they bind to their high affinity ligands (Supplementary Fig. 1). The antibodies were shown to be highly specific for their targets and to bind with high affinity. They were equivalent or superior to available commercial antibodies for the above mentioned applications.

3.3. *Protein expression of EphA1, EphA2, ephrin A1 and ephrin A5*

To assess the possible biological significance of Eph expression, we first sought to correlate EphA and ephrin A mRNA data with protein expression. Immunostaining of CRC cell lines confirmed proportionate levels of mRNA and protein expression of EphA1, EphA2, ephrin A1 and ephrin A5 (Fig. 3A). Protein expression was also examined though immunoprecipitation, Western blotting and flow cytometry. Both EphA1 and EphA2 were expressed as a single protein of the expected size (~120 kDa) (Fig. 3B) and levels correlated well with mRNA expression. To further confirm the correlation between mRNA and protein expression of EphA1 and EphA2, mRNA expression data was plotted against the mean cell fluorescence obtained through flow cytometry (Fig. 3C & D).

3.4. *Eph and ephrin expression in TissueScan Colon Tissue Q-PCR arrays*

To explore whether the data from the cell lines translated to clinical samples, colon tissue arrays were screened for nine different Eph and ephrin genes, Ephs A1, A2, A3, A7, B2, B4, and ephrins A1, A5 and B2 by Q-PCR. In keeping with the cell line data, heterogeneous EphA and ephrin A expression was observed in CRC samples. Of all the Eph genes studied, EphB4 was the most significantly expressed gene in this subset of CRCs ($p = 0.0001$) (Fig. 4). Similarly, significant levels of EphB2 and EphA1 were also seen in CRCs compared to the normal controls ($p = 0.0001$, $p = 0.005$, respectively) (Fig. 4). Although high levels of EphA2 expression were observed they were comparable between normal and CRC samples. In contrast, significant down regulation of EphA7 was noted in CRCs ($p = 0.0001$). A similar pattern was observed with EphA3, although EphA3 expression was generally low in both CRC and normal samples, but this did not reach statistical significance (Fig. 4).

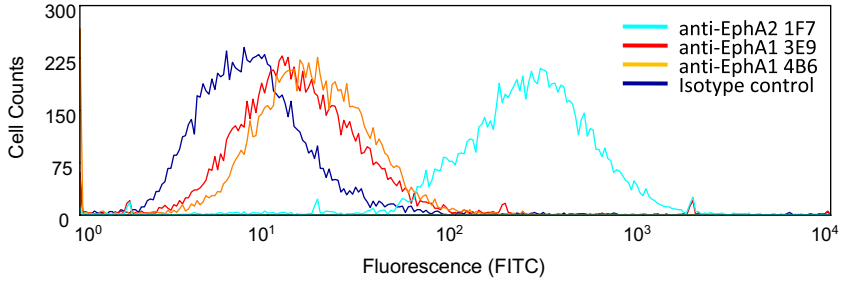

Fig. 2 – EphA1 and EphA2 protein expression in LIM1215 detected through in-house antibodies; 4B6 anti-human EphA1 IgG1, IIIE9 anti-human/mouse EphA1 IgG and IF7 anti-human/mouse EphA2 IgG1 using flow cytometry.

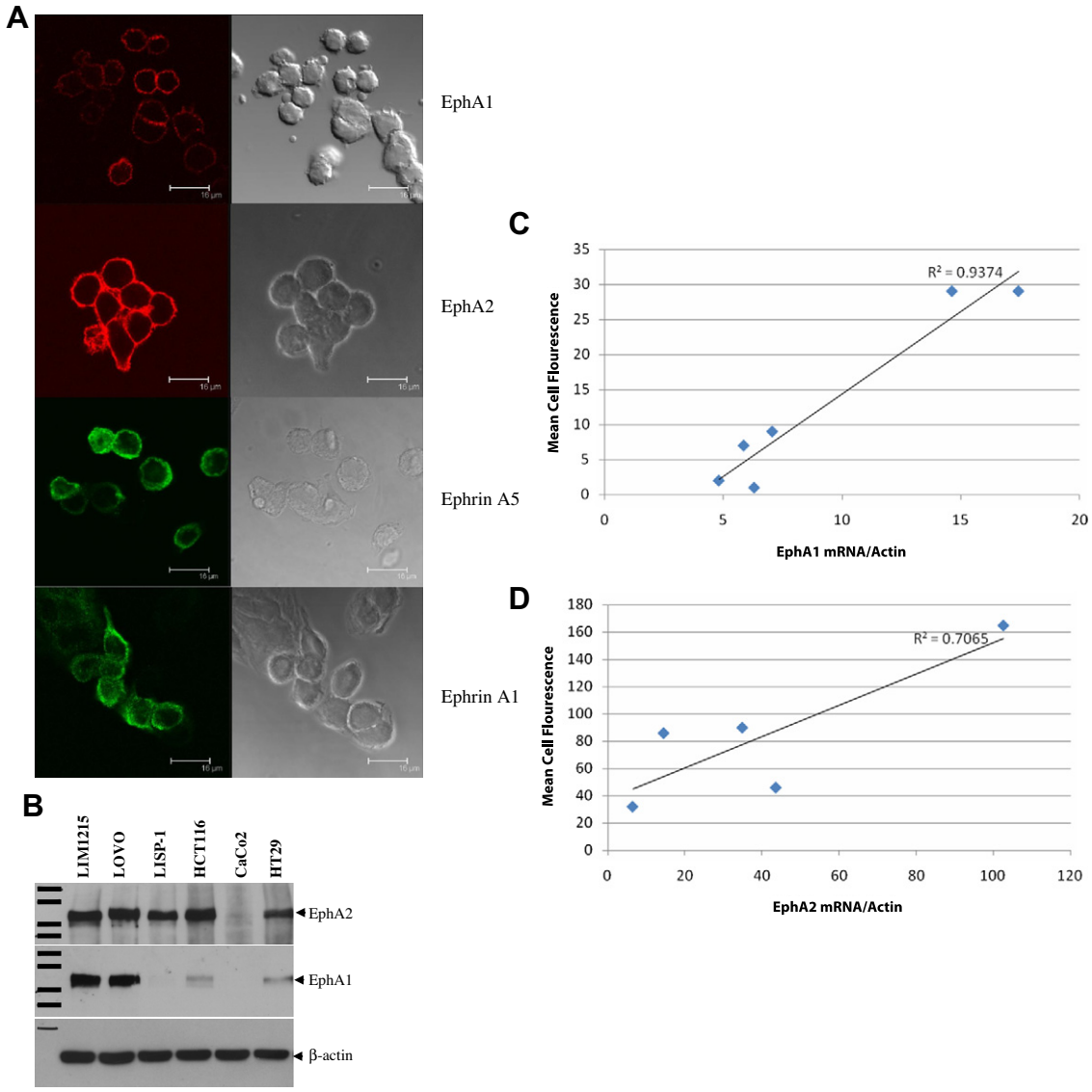

Fig. 3 – (A) Protein expression assessed through immunocytochemistry for EphA1, EphA2, ephrin A1 and ephrin A5. (B) Protein expression examined by immunoprecipitation and Western blotting. Both EphA1 and EphA2 were expressed as a single protein of the expected size (~120 kDa). (C) EphA1 mRNA expression versus Mean cell fluorescence. (D) EphA2 mRNA versus Mean cell fluorescence with the removal of the outlier LIM1215.

Ephrin A1 was the most dramatically over-expressed ephrin gene ($p = 0.0001$) compared to the normal controls. Although ephrin A5 and ephrin B2 expression were higher in CRCs compared to the normal controls this was not significant ($p > 0.05$). Eph and ephrin expression of all individual samples is shown in Supplementary Fig. 2.

Interestingly, moderate correlations were identified between EphB2 and EphB4 ($r = 0.55$; $p < 0.01$) and EphA1 and EphB4 ($r = 0.46$, $p < 0.01$). Weak correlations were also identified between EphA1 and EphB2 ($r = 0.39$; $p < 0.05$), EphA1 and ephrin A1 ($r = 0.32$; $p < 0.05$) and EphB4 and ephrin B2 ($r = 0.33$; $p < 0.05$).

Moreover it was evident that of the high expressing Eph receptors (EphA1, EphA2, EphB2, EphB4); at least one was always strongly expressed in any given CRC specimen.

3.5. *Eph and ephrin expression in paired CRC clinical samples*

Using the tissue array data, we compared a set of normal colon specimens with a collection of tumour samples. To further examine the heterogeneity of EphA and ephrin A expression we also analysed 53 cases in which both normal and CRC tissues had been obtained from each patient. Following tissue array analysis, an initial screen of EphA1, A2, A3, A7, B2, B4 and ephrin A1, A5 and ephrin B2 was performed on ten paired normal and CRC specimens.

In a subset of cases EphB2 and EphB4 were expressed at significant levels (>10 transcript/1000 ß-actin). Similarly, in some cases EphA1 and EphA2 were frequently expressed at 5–10-fold higher levels than those in normal tissues. As EphB2, EphB4 and ephrin B2 genes have been investigated

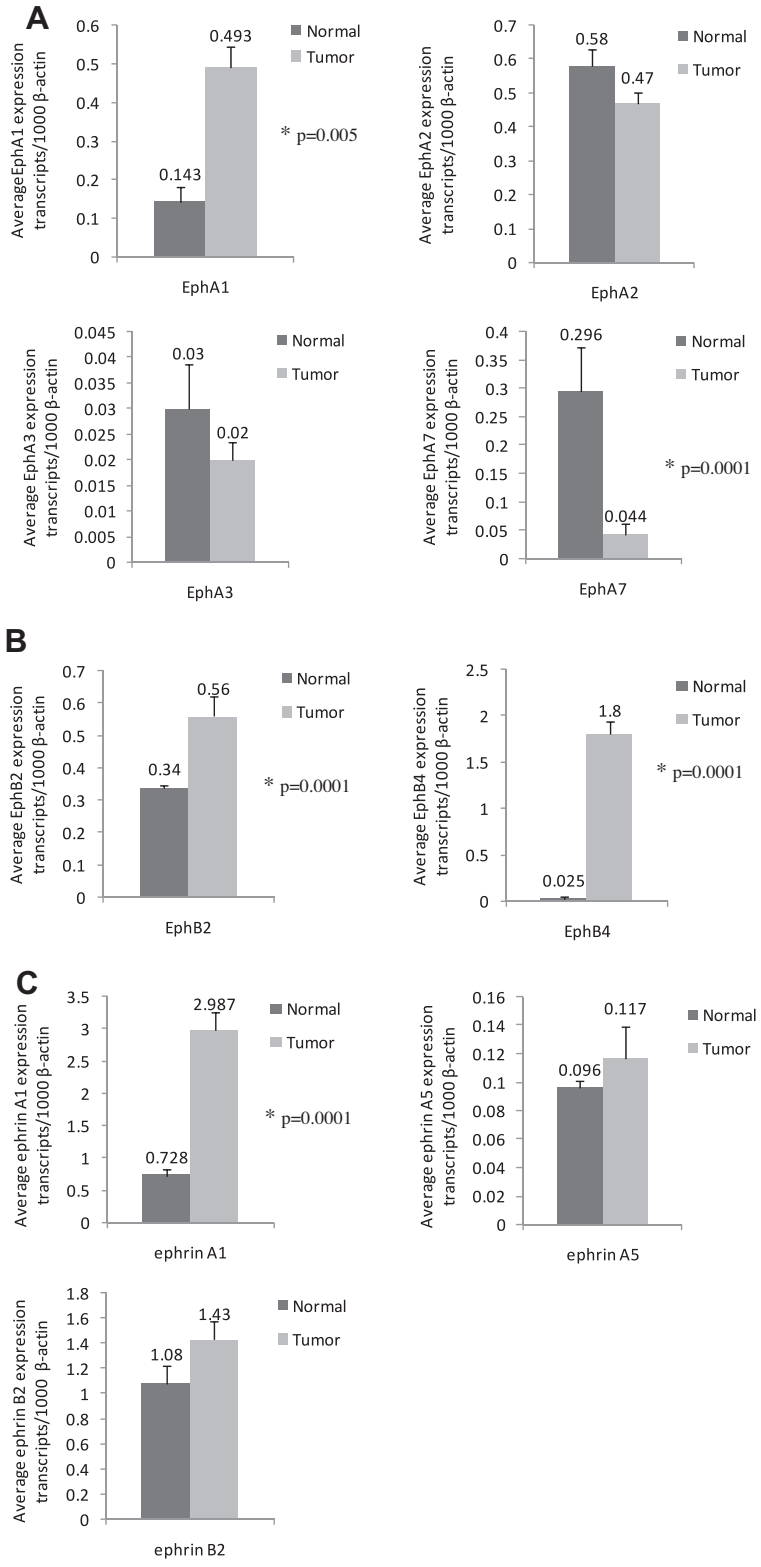

Fig. 4 – Average Eph/ephrin expression in normal colon and CRCs assessed through Colon Tissue Q-PCR arrays. (A) Average EphA1, EphA2, EphA3 and EphA7 expression. (B) Average EphB2 and EphB4 expression. (C) Average ephrin A1, ephrin A2 and ephrin B2 expression. * Indicates significant differences between normal and tumour specimens. mRNA transcript levels are normalised to 1000 copies of ß-actin quantitated by QPCR.

extensively and our data confirms previous findings, these genes were not investigated further. EphA1, A2, A3, A7, ephrin A1 and A5 were selected for further analysis in the remaining 43 paired normal and CRC samples.

3.6. Expression of EphA1, EphA2 and ephrin A1

Both EphA1 and EphA2 preferentially interact with ephrin A1 and it was notable that all three were strongly expressed in a proportion of tumours. As shown in Fig. 5, higher, in some cases >10-fold, expression of EphA1 29/53 (55%) and down regulation 20/53 (38%) was seen in a proportion of tumours compared to paired normal samples.

Similar to cell line and tissue array data, EphA2 was the most highly expressed gene in CRCs. Thirty-two of 53 (60%) tumours demonstrated higher levels of EphA2 compared to the paired normal controls. In a majority of these CRCs, EphA2 was up regulated two to five-fold compared to non-malignant control tissues. Similar to EphA1, a proportion (16/53) of CRCs also demonstrated down regulation of EphA2.

Strikingly, whilst EphA1 and EphA2 showed the greatest increase in expression relative to normal tissues, in each case there was considerable heterogeneity with some tumours having lost expression of one or more receptors. In the analysis of EphA1 and EphA2, a greater than two-fold increase in expression was observed in 53% of cases for EphA1 and 48% of cases for EphA2 with 75% of cases showing over-expression of at least one of these receptors.

Intriguingly, both EphA1 and EphA2 down-regulation was more prevalent in more advanced TNM stage III compared to TNM Stage II CRCs, most of which over-expressed EphA1 mRNA ($p = 0.02$) (Fig. 5A). This finding was independent of age, gender, tumour type and grade.[25] A similar pattern was observed with EphA2 but did not reach statistical significance (Fig. 5B).

Ephrin A1, the preferred ligand for EphA1 and a high affinity ligand for EphA2, was the highest expressed ephrin in these tumours. Twenty-seven of 53 (51%) CRCs showed increased expression of ephrin A1 compared to non-malignant control samples (Fig. 5C). The majority of these tumours (21/27, 78%) showed expression levels of two to five-fold greater than those seen in non-malignant control tissues.

3.7. Expression of EphA3, EphA7 and ephrin A5

EphA3 and EphA7 expression was considerably lower compared to EphA1 and EphA2 (Fig. 5C). Only four CRCs demonstrated increased expression of EphA3 compared to the paired normal controls. Interestingly, the majority of cases expressing EphA3 (25/53, 47%) demonstrated down regulation of this gene in the tumour.

Of the 23 cases with EphA7 expression, 17 showed decreased expression, five cases demonstrated over expression and one case had comparable levels of expression compared to the normal samples. Amongst the full cohort of samples, the analysis of EphA3 and EphA7 revealed a loss of expression in at least one of these receptors in (51/53) 96% of cases.

Expression levels of ephrin A5 were comparatively higher in CRCs compared to its high affinity receptors (Fig. 5C). Of the 37 CRCs with ephrin A5 expression, 10 showed over expression and 22 demonstrated down regulation compared to the paired normal tissue. EphA3, EphA7 and ephrin A5 expression was independent of age, gender, tumour type, grade and stage.

A strong correlation was identified between EphA2 and its high affinity ligand ephrin A1 ($r = 0.73$; $p < 0.001$). Furthermore, both EphA3 and EphA7 correlated with ephrin A5 ($r = 0.50$; $p < 0.01$ and $r = 0.45$; $p < 0.01$, respectively). There was a correlation between EphA3 and EphA7 expression ($r = 0.61$; $p < 0.01$). Weak correlations were also identified between EphA2 and EphA3 ($r = 0.33$; $p < 0.05$) and EphA2 and ephrin A5 ($r = 0.31$; $p < 0.05$).

3.8. Loss of Eph expression appears to be epigenetically mediated

In seeking a mechanism for the down regulation observed in these genes, a subsequent analysis of the human Ephs and ephrins was performed using the http://www.ebi.ac.uk/emboss/cpgplot/website (Supplementary Fig. 3). This analysis revealed that all Eph and ephrin genes examined in the current study contain CpG islands in the promoter regions (Table 1).

Based on the down regulation observed in a proportion of CRCs and the presence of CpG islands in the promoter regions, we assessed whether methylation has a direct influence on EphA and ephrin A down regulation. 5-aza-2-deoxycytidine treatment resulted in the re-expression of EphA1, EphA2, EphA3, EphA7, ephrin A1 and ephrin A5 (confirmed by Q-PCR-Supplementary Fig. 4). Although there was a slight increase in gene expression with combination treatment, this was not significant compared to 5-aza-2-deoxycytidine alone for EphA1, EphA2, ephrin A1 and ephrin A5. However, combination treatment resulted in a greater increase in re-expression for EphA3 and EphA7.

4. Discussion

In light of the confusing literature with regard to Eph gene expression, in the current study we profiled a complete survey of Eph and ephrin genes. Initial studies in cell lines revealed a heterogeneous pattern of Eph expression. However, there was a selective expression with high expression of EphA1, A2, B2 and B4 and ephrin A1 and B2 being detected in at least one of these lines. The analysis of normal colon samples showed a similar profile except that EphA3 was expressed in normal tissues but not the CRC, suggesting that expression may be in non-epithelial elements of the colon. These data are in agreement with other analyses of normal colonic tissue[13] and suggest that the tumours express a subset of the Eph and ephrin genes expressed in normal tissues.

Based on cell line and normal tissue data, the Ephs and ephrins expressed in these samples (EphA1, EphA2, EphA3, EphA7, ephrin A1, ephrin A5, EphB2, EphB4 and ephrin B2) were screened in 47 tissue array colon specimens and 10 paired normal and CRC tissues. The most important findings were the significant expression of one or more of the genes, EphA1, EphA2, ephrin A1, EphB2 and EphB4 and ephrin B2, in many cases at levels above those in normal colon, in individual CRCs and the significant down regulation of EphA7 relative to normal controls. For comparison purposes we have further assessed the microarray data available for colon adenocarcinomas in the NIH publicly available database (http://tcga-data.nci.nih.gov/tcga/) for EphA1, EphA2, EphA3, EphA7,

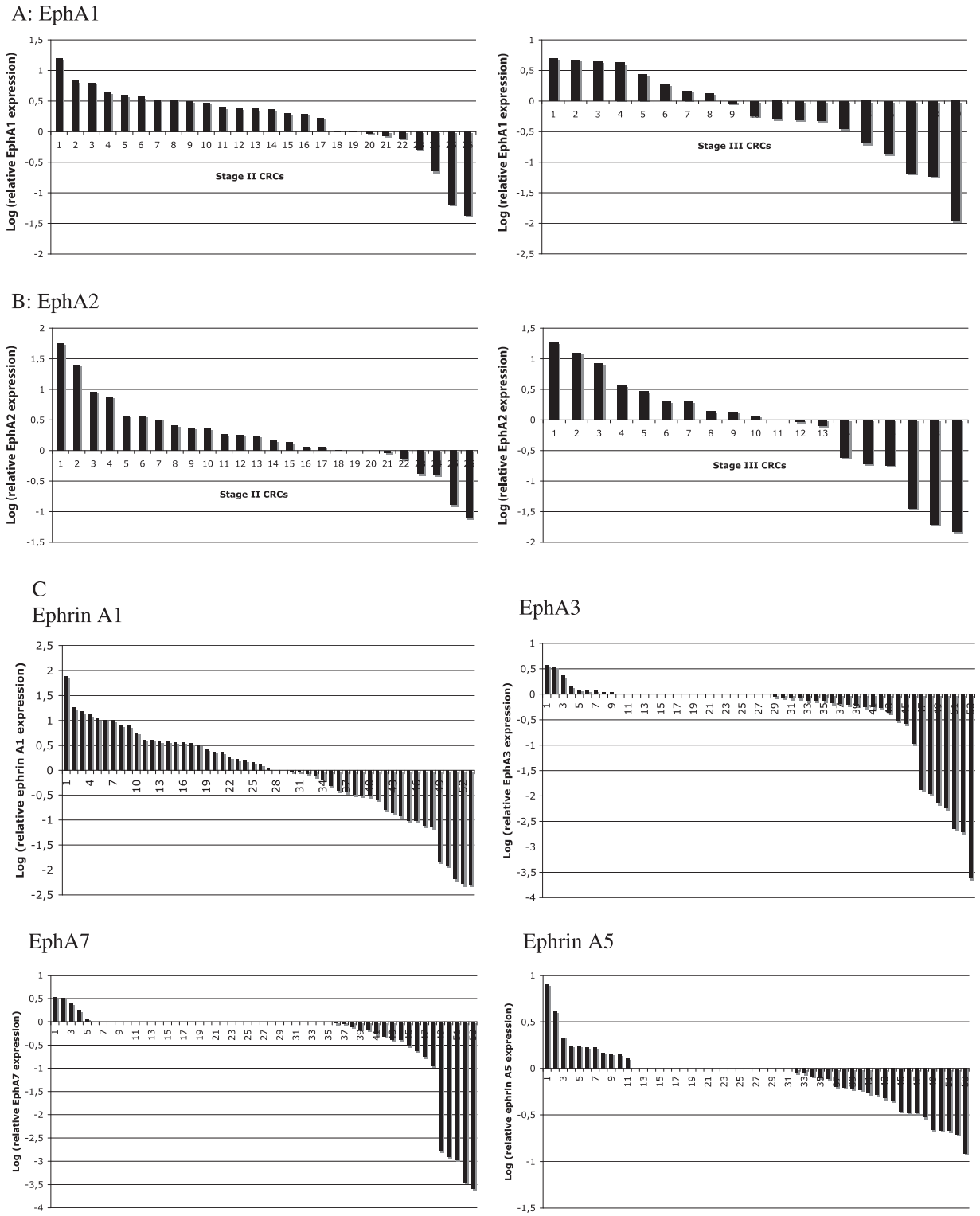

Fig. 5 – Eph/ephrin mRNA expression in paired normal and CRC specimens. (A) Relative EphA1 expression in stage II and stage III CRCs. (B) Relative EphA2 expression in stage II and stage III CRCs. (C) Relative ephrin A1, EphA3, EphA7 and ephrin A5 in all CRCs. Eph/ephrin transcript levels are normalised to 1000 copies of ß-actin quantitated by QPCR. The pfaffl method of quantification was used for data analysis of the paired normal and tumour samples. Expression levels in CRCs are represented relative to the paired normal sample.

EphB2 and EphB4 (Supplementary Fig. 5). No data was available for the ephrins in this database. Albeit from a less sensitive technique similar to our data, these data suggest that EphA1, EphA2, EphA3, EphA7, EphB2 and EphB4 are

Table 1 – Summary of CpG islands in the promoter regions of the Eph and ephrin genes examined.

Gene	CpG islands	Length	Location
EphA1	1	251 bp	Starts at the 5 UTR and spans exon 1 and intron 1
EphA2	1	779 bp	Starts at the 5 UTR and spans exon 1 and intron 1
EphA3	2	1st-402 bp	Intron 1
		2nd-419 bp	Intron 16
EphA7	1	404 bp	Intron 1
EphB2	1	1400 bp	Starts at the 5 UTR and spans exon 1 and intron 1
EphB4	1	760 bp	Starts at the 5 UTR and spans exon 1
Ephrin A1	1	414 bp	Starts at the 5 UTR and spans exon 1
Ephrin A5	1	730 bp	Starts at the 5 UTR and spans exon 1 and intron 1
Ephrin B2	2	1st-625 bp	Starts at the 5 UTR and spans exon 1
		2nd-781 bp	Spans exon 1 and intron 1

up-regulated in a subset of CRCs whilst down regulated in a proportion of CRCs.

In agreement with the findings in this study, increased expression of EphB2, EphB4 and ephrin B2 expression has been previously reported in CRCs.[9,26,27] We found that, as with the cell lines, there was considerable heterogeneity of expression of all of these genes with some tumours showing significant (>five-fold) over expression whilst other comparable tumours revealed much lower expression than normal tissues. This heterogeneity was more dramatically shown in the analysis of paired normal and CRC samples (Fig. 5), which again showed almost uniform down regulation of EphA7 but large variability in EphA1 and EphA2 expression. A clue to the mechanism underlying this variability was the observation that more advanced (TNM Stage III) CRCs showed a higher frequency of loss of expression than earlier stage lesions. We have already shown that EphA1 over-expression is commonly seen in locally invasive CRC but that down-regulation is more frequent in metastatic CRC, in many cases mediated through epigenetic gene silencing.[25] Here we show the same pattern of expression for EphA2 and a similar phenomenon has also been described for EphB2, where the loss of expression was associated with cancer progression,[7,8,28] and higher EphB2 expression associated with prolonged survival.[28]

The loss of EphA7 has also been shown to be due to promoter methylation[12] suggesting that epigenetic mechanisms may be involved in progressive loss of expression of a subset of Eph/ephrin genes. Consistent with this idea we show that all Eph and ephrin genes appear to contain CpG islands in their promoter regions and thus could be subject to epigenetic silencing. Moreover, we show that treatment with the demethylating agent 5′-aza-deoxycytidine resulted in re-expression of Eph genes in cell lines.

By analysing all Eph genes, a consistent picture emerges from these studies. This shows that all CRC cases we have analysed express at least one of the Eph genes found in normal colon, often at levels significantly greater than that seen in normal tissues. The redundancy of Eph gene function would thus be consistent with a model in which, as the CRC evolves and progresses, one Eph gene becomes dominant whilst other members are lost through epigenetic or genetic mechanisms. Thus, the multiplicity of Eph expression in early lesions with concomitant high levels of Eph signalling is gradually replaced by restricted Eph expression and reduced signalling in more advanced CRCs. This notion that reduced expression, and hence a loss of Eph signalling, might favour progression is supported by an animal model in which enforced EphB expression could suppress CRC.[29]

In terms of the retained Eph expression it was of interest that correlations were identified between expression of EphA1 receptor and its high affinity ligand, ephrin A1 and of the EphB4 receptor and its preferred ligand ephrin B2. EphA1 and EphB4 are the Eph receptors which show the least redundancy of ephrin interaction.[30] Thus, the highly expressed Eph might be selected during tumour evolution, in part, by co-expression of a cognate ephrin ligand on the tumour such that Eph signalling is mediated by tumour cell-tumour cell interactions and is no longer dependent on ephrin expression by non-malignant supporting cells.

Whilst EphB receptors have been shown to be highly expressed in some CRCs this report shows that the EphA RTKs, EphA1 and EphA2 in particular, are expressed at high levels in many CRCs and thus may be potential targets for therapy in this disease. Eph proteins have been identified as therapeutic targets in cancer, both as direct anti-cancer and anti-angiogenesis agents, and several therapeutic candidates are in advanced pre-clinical or early clinical assessment. This makes understanding the heterogeneity of Eph expression during CRC progression, and potentially in other epithelial tumours, of critical importance. Whilst therapies targeting the high expression in early phase disease seem logical, the loss of expression in advanced disease poses the risk that targeted therapies may be either ineffective or even selected for loss of expression and even contribute to disease progression. Our data suggest that individual CRCs need to be phenotyped to determine which Eph genes are highly expressed and hence potential targets. It follows that a cocktail of specific therapies targeting a number of Eph receptors is required to cover all CRCs. Specific antibodies such as those developed for EphA1 in this study are potential therapy candidates in CRC.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.07.003.

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