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

Nirmitha I. Herath [a,*], *Mark D. Spanevello* [a], *James D. Doecke* [b], *Fiona M. Smith* [a], *Celio Pouponnot* [c], *Andrew W. Boyd* [a,d]

[a] *Leukaemia Foundation Research Laboratory, The Queensland Institute of Medical Research, 300 Herston Road, Brisbane, Queensland 4029, Australia*

[b] *Cancer and Population Studies, The Queensland Institute of Medical Research, 300 Herston Road, Brisbane, Queensland 4029, Australia*

[c] *Cellular Regulation and Oncogenesis Laboratory, The Curie Institute, Orsay, France*

[d] *Department of Medicine, The University of Queensland, Brisbane, Queensland 4029, Australia*

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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

* *Corresponding author:* Tel.: +33 1 6986 3040; fax: +33 1 6986 3051.
E-mail address: nirmitha.herath@curie.u-psud.fr (N.I. Herath).

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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