Short Communication

Tissue microarrays characterise the clinical significance of a VEGF-A protein expression signature in gastrointestinal stromal tumours

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A tissue microarray analysis of 22 proteins in gastrointestinal stromal tumours (GIST), followed by an unsupervised, hierarchical monothetic cluster statistical analysis of the results, allowed us to detect a vascular endothelial growth factor (VEGF) protein overexpression signature discriminator of prognosis in GIST, and discover novel VEGF-A DNA variants that may have functional significance.

British Journal of Cancer (2007) 96, 776–782. doi:10.1038/sj.bjc.6603551 www.bjcancer.com

Published online 13 February 2007
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Keywords: gastrointestinal stromal tumours; VEGF; tissue microarrays

The clinical behaviour of gastrointestinal stromal tumours (GIST) is notoriously difficult to predict. The prognostic and therapeutic significance of KIT mutations is somewhat contradictory (Ernst et al., 1998; Lasota et al., 1999; Moskaluk et al., 1999; Taniguchi et al., 1999; Lasota et al., 2000; Hirota et al., 2001; Wardelmann et al., 2002; Koay et al., 2005). Therefore, it appears that new molecular indicators of prognostication are needed. Tissue microarrays (TMA) is a high-throughput method for the analysis of large numbers of formalin-fixed, paraffin-embedded (FFPE) materials with minimum cost and effort (Konen et al., 1998). Here, we applied the TMA technology to analyse protein expression in GIST.

The results were analysed with an unsupervised, hierarchical monothetic cluster statistical method. Those biomarkers with strong clinical significance were tested for mutation status by both PCR-denaturing high performance liquid chromatography (DHPLC) and direct sequencing. By doing so, we identified a VEGF-A protein overexpression signature as a statistically significant predictor of malignancy, discovered VEGF-A ligand DNA variants in GIST, and provided other possible targets in future design of anti-VEGF-directed therapy against GIST.

MATERIALS AND METHODS

We used 50 archival paraffin blocks (Department of Pathology, National University Hospital, Singapore), including 15 cases of GIST with a benign outcome, 17 with a malignant outcome (13 primary neoplasms and four metastases), 10 with no available clinical follow-up, and eight gastrointestinal mesenchymal neoplasms other than GIST, such as leiomyoma (n = 5), leiomyosarcoma, neurofibroma and schwannoma (one of each). The mean clinical follow-up was of 39 months. The overall clinicopathological characteristics are summarised in Table 3. No chemo or radiotherapy was given to these patients. All the gross and histopathological parameters classically associated with malignant potential were analysed. The findings were similar to those reported in other series (data not shown) and, in themselves, are considered insufficient for single-case prognostication in the clinical setting.

After case review for diagnostic confirmation, the TMA was constructed as reported elsewhere (Zhang et al., 2003a; Salto-Tellez et al., 2004). The 22 antibodies used are 34 BE12, AE 1/3, Bcl-2, CAM 5.2, CD10, CD117, CD34, c-erbB2, CK7, CK20, Desmin, Flk-1, Flt-1, Hep Par1, Ki-67, MNF 116, p53, PCNA, S100, SMA, VEGF-A and Vimentin. Table 4 indicates the antibodies and their technical specifications. In general, these antibodies can be divided into several groups: diagnostic markers, antibodies expressed in a specific differentiation pathway relevant to GIST, proliferative or apoptosis-related markers, angiogenic proteins, and others that may have been associated before with prognostic significance in GIST. The interpretation of the IHC staining results for TMA was confirmed by three independent observers (NME, LCK and MST). Results were interpreted based on previous published experience for each individual antibody.

The concordance between TMA and full sections, tested for five antibodies (Table 5) ranged from 92–100% in five of six antibodies, excluding S100 (71%), in concordance with previous published results (Zhang et al., 2003a).

The 28 FFPE cases with available clinical follow-up were the subject of genomic DNA extraction (GENTRA DNA Purification Kit – Gentra, Minneapolis, MN, USA), according to the manufacturer’s instruction. Mutation analysis was performed by PCR-DHPLC analysis. Briefly, DNA was amplified in 25 µl reactions containing 2 µl DNA template, 1 µl of each forward and
reverse primers (10 μM each), 0.5 μl of 10 mM dNTP, 0.2 μl FastStart Taq (Roche, Mannheim, Germany), and 1 × PCR reaction buffer with MgCl₂. Primer sequences and cycling conditions are indicated in Tables 6 and 7. The PCR product (8 μl) was denatured at 95°C for 5 min followed by gradual re-annealing to room temperature for over a period of 1 h. DHPLC was performed using a fully automated WAVE 3500HT system (Transgenic, Omaha, NE, USA). The cooled samples were automatically injected into a

| Case no. | VEGF-A | Flt-1 | Flik-1 | SMA | CD117 | Vimentin | CD34 | Desmin | S100 |
|----------|--------|-------|--------|-----|-------|----------|------|--------|------|
| Group 1: VEGF-A expression |
| B2       |        |       |        |     |       |          |      |        |      |
| M(M6)    |        |       |        |     |       |          |      |        |      |
| M4       |        |       |        |     |       |          |      |        |      |
| M3       |        |       |        |     |       |          |      |        |      |
| M(M5)    |        |       |        |     |       |          |      |        |      |
| B1       |        |       |        |     |       |          |      |        |      |
| M5       |        |       |        |     |       |          |      |        |      |
| NF       |        |       |        |     |       |          |      |        |      |
| M9       |        |       |        |     |       |          |      |        |      |
| M10      |        |       |        |     |       |          |      |        |      |
| M1       |        |       |        |     |       |          |      |        |      |
| B5       |        |       |        |     |       |          |      |        |      |
| B8       |        |       |        |     |       |          |      |        |      |
| U1       |        |       |        |     |       |          |      |        |      |
| B13      |        |       |        |     |       |          |      |        |      |
| M7       |        |       |        |     |       |          |      |        |      |
| M8       |        |       |        |     |       |          |      |        |      |
| M11      |        |       |        |     |       |          |      |        |      |
| M12      |        |       |        |     |       |          |      |        |      |
| M13      |        |       |        |     |       |          |      |        |      |
| U2       |        |       |        |     |       |          |      |        |      |
| M(M9)    |        |       |        |     |       |          |      |        |      |
| M(M2)    |        |       |        |     |       |          |      |        |      |
| SCH      |        |       |        |     |       |          |      |        |      |
| Group 2: VEGF-A lack of expression |
| B3       |        |       |        |     |       |          |      |        |      |
| B4       |        |       |        |     |       |          |      |        |      |
| B7       |        |       |        |     |       |          |      |        |      |
| U3       |        |       |        |     |       |          |      |        |      |
| B6       |        |       |        |     |       |          |      |        |      |
| U4       |        |       |        |     |       |          |      |        |      |
| B10      |        |       |        |     |       |          |      |        |      |
| B11      |        |       |        |     |       |          |      |        |      |
| B12      |        |       |        |     |       |          |      |        |      |
| B15      |        |       |        |     |       |          |      |        |      |
| B14      |        |       |        |     |       |          |      |        |      |
| U5       |        |       |        |     |       |          |      |        |      |
| M6       |        |       |        |     |       |          |      |        |      |
| U6       |        |       |        |     |       |          |      |        |      |
| U7       |        |       |        |     |       |          |      |        |      |
| M2       |        |       |        |     |       |          |      |        |      |
| U2       |        |       |        |     |       |          |      |        |      |
| B9       |        |       |        |     |       |          |      |        |      |
| LM1      |        |       |        |     |       |          |      |        |      |
| LM5      |        |       |        |     |       |          |      |        |      |
| LM2      |        |       |        |     |       |          |      |        |      |
| LM3      |        |       |        |     |       |          |      |        |      |
| LM4      |        |       |        |     |       |          |      |        |      |
| Group 3: Smooth muscle signal |

Green indicates antibody expression, whereas red indicates lack of expression. The tumours are divided into four clinically relevant groups – GISTs that are clinically benign, malignant and of unknown clinical outcome; and non-GISTs: LMS – leiomyosarcoma, LM – leiomyoma; NF – neurofibroma; SCH – schwannoma. Cases with brackets are metastasis from the original tumour, the latter indicated within brackets, for example, M(M2) denotes the liver metastasis from case M2. Those antibodies not included in the table (34 βE12, AE 1/3, CK7, CK20 and Hep Par1) were universally negative for all the samples of the study.
### Table 2  Protein expression and sequence status of VEGF and KIT in malignant and benign GIST samples

| Case | VEGF | KIT | VEGF | VEGF | VEGF | KIT |
|------|------|-----|------|------|------|-----|
|      | IHC  | IHC | Exon 1 | Exon 3 | Exon 4 | Exon 11 |
| benign | +   | +   | +     | +     | +     | 550A:deletion 27bp |
| 2    | +   | +   | +     | +     | +     | 559C:deletion 6bp |
| 3    | +   | +   | +     | +     | +     | 557A:deletion 27bp |
| 4    | +   | +   | +     | +     | +     | 550A:deletion 27bp |
| 5    | +   | +   | +     | +     | 557T:deletion 6bp |
| 6    | +   | +   | +     | +     | 558G:deletion 3bp |
| 7    | +   | +   | +     | +     | 558G:deletion 3bp |
| 8    | +   | +   | +     | +     | +     |
| 9    | +   | +   | +     | +     | +     |
| 10   | +   | +   | +     | +     | +     |
| 11   | +   | +   | +     | +     | +     |
| 12   | +   | +   | +     | +     | +     |
| 13   | +   | +   | +     | +     | +     |
| malignant | +   | +   | +     | +     | +     |
| 1    | +   | +   | +     | +     | +     |
| 2    | +   | +   | +     | +     | +     |
| 3    | +   | +   | +     | +     | +     |
| 4    | +   | +   | +     | +     | +     |
| 5    | +   | +   | +     | +     | +     |
| 6    | +   | +   | +     | +     | +     |
| 7    | +   | +   | +     | +     | +     |
| 8    | +   | +   | +     | +     | +     |
| 9    | +   | +   | +     | +     | +     |
| 10   | +   | +   | +     | +     | +     |
| 11   | +   | +   | +     | +     | +     |
| 12   | +   | +   | +     | +     | +     |
| 13   | +   | +   | +     | +     | +     |
| 14   | +   | +   | +     | +     | +     |
| 15   | +   | +   | +     | +     | +     |

+= expression, -= no expression. Sequence variants are denoted as 'codon followed by nucleotide position (A = 1st, B = 2nd, C = 3rd): nucleotide change (protein change)'.
Non-coding variants are denoted as 'IVS, exon, nucleotides from exon start: nucleotide change'.

### Table 3  Characteristics of benign (B) and malignant (M) GISTs

| No | Age | Site     | Size (mm) | Cell type | Mitoses (50 HPF) | SMA % +ve | CD34 % +ve | CD117 % +ve | Status (months) | Metastases/recurrence |
|----|-----|----------|-----------|-----------|------------------|-----------|-------------|--------------|-----------------|------------------------|
| B1 | 45  | Duod    | 20 s      | Spindle   | 2                | 0         | 0           | 100          | aned (76)       | Nil                    |
| B2 | 39  | Gastric | 70 m      | Mixed     | 1.5              | 0         | 100         | 80           | aned (124)      | Nil                    |
| B3 | 45  | Gastric | 10 s      | Spindle   | 0                | 0         | 100         | 95           | aned (24)       | Nil                    |
| B4 | 46  | Gastric | 27 s      | Spindle   | 1                | 0         | 100         | 100          | aned (20)       | Nil                    |
| B5 | 53  | Gastric | 29 m      | Spindle   | 0                | 0         | 80          | 85           | aned (24)       | Nil                    |
| B6 | 69  | Gastric | 35 s      | Spindle   | 1                | 10        | 100         | 40           | aned (87)       | Nil                    |
| B7 | 71  | Gastric | 90 s      | Spindle   | 1                | 0         | 100         | 100          | aned (3)        | Nil                    |
| B8 | 77  | Gastric | 45 s      | Spindle   | 1                | 0         | 100         | 80           | aned (68)       | Nil                    |
| B9 | 42  | Gastric | 50 s      | Spindle   | 3.5              | 0         | 0           | 0            | aned (13)       | Nil                    |
| B10| 50  | Gastric | 100 s     | Spindle   | 1                | 0         | 90          | 30           | aned (10)       | Nil                    |
| B11| 62  | Gastric | 6 s       | Spindle   | 15               | 0         | 100         | 100          | aned (1)        | Nil                    |
| B12| 87  | Gastric | 25 s      | Spindle   | 0                | 0         | 100         | 100          | aned (6)        | Nil                    |
| B13| 87  | Gastric | 7 s       | Spindle   | 3                | 0         | 100         | 100          | aned (12)       | Nil                    |
| B14| 47  | Pelvic  | 60 s      | Spindle   | 4                | 0         | 100         | 100          | aned (83)       | Nil                    |
| B15| 49  | Jejunal | 45 s      | Spindle   | 2                | 0         | 0           | 100          | aned (60)       | Nil                    |
| M1 | 67  | Colon   | 90 s      | Spindle   | 15               | 0         | 100         | 0            | dod (21)        | LR                     |
| M2 | 37  | Duodenal| 60 m      | Spindle   | 4.5              | 0         | 70          | 50           | dod (89)        | Liver                  |
| M3 | 36  | Gastric | 180 s     | Epithelioid| 62.5             | 0         | 100         | 100          | dod (17)        | Liver                  |
| M4 | 52  | Gastric | 190 s     | Spindle   | 7.5              | 0         | 100         | 100          | dod (36)        | No data                |
| M5 | 59  | Gastric | 70 e      | Epithelioid| 10               | 0         | 0           | 0            | dod (72)        | Liver, bones, abdominal nodes |
| M6 | 71  | Gastric | 170 e     | Epithelioid| 24               | 0         | 100         | 100          | dod (103)       | Omentum, LR           |
| M7 | 41  | Gastric | 100 e     | Epithelioid| 26               | 0         | 100         | 75           | dod (43)        | Retroperitoneum        |
| M8 | 48  | Gastric | 35 s      | Epithelioid| 24.5             | 0         | 100         | 70           | dod (27)        | Peritoneum            |
| M9 | 48  | Gastric | 150 s     | Epithelioid| 31               | 0         | 100         | 70           | dod (22)        | Liver, spleen          |
| M10| 68  | Gastric | 110 s     | Epithelioid| 113.5            | 0         | 100         | 85           | dod (7)         | Liver, LR             |
| M11| 73  | Gastric | 60 s      | Epithelioid| 66.5             | 0         | 100         | 100          | dod (8)         | No data                |
| M12| 65  | Jejuno-ileal | 90 s | Epithelioid| 52               | 45        | 100         | 100          | dod (15)        | Peritoneum            |
| M13| 33  | Rectal  | 60 s      | Spindle   | 0.5              | 2.5       | 100         | 70           | duc             | Liver, bone, para-aortic nodes, lungs |

B = Benign cases; M = Malignant cases; s = spindle cell type; e = epithelioid cell type; m = mixed epithelioid and spindle cell type; aned = alive with no evidence of disease; awd = alive with disease; dod = died of disease; duc = died of unrelated causes; LR = local recurrence.
DNAseq cartridge (Transgenomic) and eluted at a flow rate of 0.9 ml min⁻¹ through a linear gradient of acetonitrile containing 0.1 M triethylammonium acetate (TEAA). Buffer A (0.1 M TEAA solution) and buffer B (0.1 M TEAA with 25% acetonitrile solution) concentrations and oven temperatures for optimal heteroduplex separation under partially DNA denaturation was determined using the WAVE Navigator software followed by empirical adjustment. Amplicons from the HeLa cell line were included in each run as a wild-type reference.

Samples showing a DHPLC aberrant elution profile were re-amplified and sequenced in both directions. Direct sequencing was performed on the ABI PRISM Model 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA), using the same primers as were used for amplification. Sequencing reactions were conducted with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions.

The monothetic cluster analysis was carried out as reported elsewhere (Zhang et al, 2003b) Significance tests included the student’s unpaired t-test (2-tailed) for numerical variables and the Fisher’s exact probability test for categorical variables. Significance value for P was taken to be P<0.05.

RESULTS

VEGF protein expression signature and its prognostic significance in GIST

Table 1 shows the whole protein expression results. Figure 1 shows the cluster diagram obtained upon monothetic hierarchical cluster analysis, including IHC of representative cases. From the cluster analysis, two main groups emerged, based on reactivity for the VEGF-A ligand antibody. Group 1 includes all the VEGF-A ligand expressing cases; out of the 20 GISTs with known clinical outcome, 15 were malignant (75%). In group 2 (VEGF-A negative), only 2/11 of the cases (18%) had a malignant outcome. The difference was statistically significant (P=0.003). Within group 2, the two malignant cases are further subclassified into a cluster arm, which is positive for flt-1, a receptor for VEGF. Hence, all 17/17 malignant cases were positive for either VEGF-A ligand or the VEGF-A receptor, flt-1, as compared to 8/15 of the benign cases (P=0.006). Indeed, concomitant expression of VEGF ligand and VEGF receptor

Table 4 Antibodies used

| Antibody   | Type       | Source                          | Dilution |
|------------|------------|---------------------------------|----------|
| 34 BE12    | Monoclonal | Dako, Glostrup, Denmark        | 1:500    |
| AE 1/3     | Monoclonal | Dako, Glostrup, Denmark        | 1:1000   |
| Bcl-2      | Monoclonal | Dako, Glostrup, Denmark        | 1:200    |
| CAM 5.2    | Monoclonal | Becton-Dickinson, San Jose      | 1:120    |
| CD10       | Monoclonal | Novocastra, Newcastle, UK       | 1:200    |
| CD117      | Polyclonal | Dako, Denmark                  | 1:1000   |
| CD34       | Monoclonal | Dako, Glostrup, Denmark        | 1:1000   |
| c-erbB2    | Monoclonal | Signet Laboratories Inc.        | 1:1200   |
| CK7        | Monoclonal | Dako, Glostrup, Denmark        | 1:2000   |
| CK20       | Monoclonal | Neomarker, Fremont, CA, USA    | 1:1200   |
| Desmin     | Monoclonal | Neomarker, Fremont, CA, USA    | 1:500    |
| Fli-1      | Monoclonal | Santa Cruz Biotechnology        | 1:500    |
| Ft-1       | Monoclonal | Santa Cruz Biotechnology        | 1:1000   |
| Hep Par1   | Monoclonal | Dako, Glostrup, Denmark        | 1:500    |
| Ki-67      | Monoclonal | Dako, Glostrup, Denmark        | 1:1000   |
| MNF 116    | Monoclonal | Dako, Glostrup, Denmark        | 1:1000   |
| p53        | Monoclonal | Dako, Glostrup, Denmark        | 1:1000   |
| PCNA       | Polyclonal | Dako, Glostrup, Denmark        | 1:1000   |
| S100       | Polyclonal | Dako, Glostrup, Denmark        | 1:10000  |
| SMA        | Monoclonal | Dako, Glostrup, Denmark        | 1:10000  |
| VEGF-A     | Monoclonal | Santa Cruz Biotechnology        | 1:1500   |
| Vimentin   | Monoclonal | Dako, Glostrup, Denmark        | 1:10000  |

Table 5 Comparison of results of TMA vs full section analysis

| Antibody | SMA | Vm | CAMS5.2 | CD117 | CD34 | S100 |
|----------|-----|----|---------|-------|------|------|
| Full sections | + | 14 | 47 | 3 | 39 | 37 | 17 |
| – | 37 | 4 | 48 | 12 | 14 | 34 |
| TMA | + | 14 | 47 | 1 | 35 | 34 | 4 |
| – | 37 | 4 | 50 | 16 | 17 | 47 |
| Disagree | 4 | 100 | 96 | 94 | 71 |
| Concordance % | 92 | 100 | 96 | 92 | 71 |

Table 7 VEGF-A PCR conditions

| Exon | Forward primer | Reverse primer | Size (bp) | Temperature (°C) | Oven temperature (°C) | Buffer concentration (%B) |
|------|----------------|----------------|-----------|-----------------|-----------------------|--------------------------|
| 1    | GGGGAGGAGGAGGATGACG | GCACCTTAAGTGCACTCAGGG | 324 | 60 | 66.6 | 55.4 |
| 2    | CTGTGCTGAGGAGGAGTGGT | AAGGAAATGGGCACTGGACCC | 314 | 65 | 63.0 | 47 |
| 3    | GCAGTCACTTCTTCTGTCG | GTGCCCAAGGTTAACCTCACC | 234 | 65 | 61.8 | 55.1 |
| 4    | GTTGTCCATCGGAGTATC | TAAACCTGGACGGTTACAGGG | 210 | 65 | 60.9 | 46.3 |
| 5    | TACCATCTACCATCATCC | ACAGAGGTAGGACGACGAGG | 161 | 65 | 60.7 | 39 |
| 6    | CCGTGTCCTACCATCACCTC | GAGGCTCCAGGGAGTACG | 188 | 65 | 60.8 | 41 |
| 7    | CAGCTGGGGAGAGTTAGGG | TCGCTCGCTACCTCTTTC | 313 | 65 | 59.8 | 55.1 |
represents a VEGF-A protein expression signature in GIST with obvious clinical significance. Lastly, proliferation and oncogenic-related markers PCNA, Ki-67/MIB, bcl-2 and p53 showed no statistically significant preference in reactivity for malignant GISTs ($P > 0.05$). The fact that all the smooth muscle lesions included in the analysis are clustering in a separate group (group 3) is a measure of the robustness of this analytical approach.

New VEGF-A variants are discovered as a result of mutation analysis

Those GIST samples with known clinical follow-up underwent genomic analysis. In view of the evidence of KIT mutations in GIST and their possible prognostic value (as well as their relation to imatinib therapeutic response) (Lasota et al, 1999; Heinrich et al, 2003), exons 9, 11, 13, 17 of KIT (which are those related to prognosis in the literature) were analysed in the same methodological manner. The results are summarised in Table 2. Variants identified included non-coding IVS1–7:C→T changes in five (18%) samples (Figure 2), IVS4–28:C→T changes in seven (25%) samples and coding codon 48A:G→T (Q→H) and codon 91A:G→A (G→D) changes in one sample each (Table 2). A total of 12 (43%) cases had variants in KIT, all in exon 11 (Figure 3). VEGF IVS4–28:C→T variants were more frequent in samples with low (5/7, 71%) than high (2/7, 29%) VEGF-A expression. The VEGF codon 48 and 91 mutants were present in samples with high

**Figure 1** In red are the study cases with malignant behaviour, in blue are those cases with benign behaviour; cases without available follow-up and non-GISTs are in black. The TMA immunohistochemistry results are included. The asterisk indicates cases reflected in the photomicrographs. Other abbreviations are similar to those described in Table 1. VEGF1 is equivalent to VEGF-A in this figure.
the associations between sequence variants and the expression of their respective proteins, or with the presence of each other, were significant, presumably due to the limited number of samples in this series. The only parameter significantly associated with malignancy in these selected 28 cases was, as expected, VEGF-A protein expression ($P = 0.020$). Of interest, there was no association with exon 11 KIT mutations and survival in our series.

**DISCUSSION**

The uncertain prognosis of GIST, both before (Nilsson et al, 2005) and after (Kosmadakis et al, 2005) imatinib treatment, indicate the need for the search of other molecular prognostication biomarkers.

GIST are highly vascularised neoplasms and VEGF-A is a major angiogenic therapeutic target (Ferrara and Kerbel, 2005). Recently, anti-VEGF-A therapy has been successful in the treatment of GIST (Marx, 2005), with drugs such as Sutent and Sorafenib. Our results indicate that a combined VEGF-A ligand-receptor protein expression signature is a determinant of clinical behaviour in GIST. This is obvious in our study because (a) there is a relation between protein overexpression of the VEGF-A ligand and Flt-1 proteins and benign/malignant behaviour; (b) novel variants in the VEGF-A ligand gene are characterised, some of which appear related to a malignant behaviour (such as VEGF-A exon 3); and (c) in general, these VEGF-ligand variants localise to areas of the VEGF protein with functional significance. The role of Flt-1 in this context is unclear; it could be related to the induction of metalloproteinases (Hiratsuka et al, 2002), or to chemotactic signals (Wey et al, 2005).

The role of the detected VEGF-A ligand variants in protein overexpression and GIST tumorigenesis can only be a matter of speculation, based on the scant information available. The IVS4–28:C→T variant is also identified in phenotypically normal gastrointestinal tissue, thus may not be relevant. The two other variants, however, may have functional implications. The IVS1–7:C→T variant lies within a GC box that binds the transcriptional repressor protein methyl CpG binding protein-2 (Lapchak et al, 2004), and was found to be associated with higher levels of VEGF mRNA in colorectal cancer (Yamamori et al, 2004), increasing the risk of liver metastasis and worsening its prognosis. In addition, two missense mutations (unreported to date) were discovered in exon 3, coding codon 48A:G→T (Q→H) and codon 91A:G→A (G→D), both in malignant GIST and both showing VEGF-A ligand protein overexpression. In any case, the evidence points to the novel hypothesis that VEGF-A ligand mutations may play a role if the biology and prognosis of GIST.

There has been a previous suggestion that VEGF-A ligand protein expression may be related to prognosis (Takahashi et al, 2003). However, the strength of our unsupervised hierarchical cluster analysis, comparing the expression of an antibody in the context of another 21 biomarkers, delineates ‘biological groups’ and establishes more complete ‘prognostic signatures’, which in our study, shown the importance of including protein expression of both VEGF-A ligand and Flt-1 receptor in the characterisation of malignant behaviour.

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