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Alternative splicing of TIA-1 in human colon cancer regulates VEGF isoform expression, angiogenesis, tumour growth and bevacizumab resistance

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ABSTRACT

The angiogenic capability of colorectal carcinomas (CRC), and their susceptibility to anti-angiogenic therapy, is determined by expression of vascular endothelial growth factor (VEGF) isoforms. The intracellular protein T-cell Intracellular Antigen (TIA-1) alters post-transcriptional RNA processing and binds VEGF-A mRNA. We therefore tested the hypothesis that TIA-1 could regulate VEGF-A isoform expression in colorectal cancers. TIA-1 and VEGF-A isoform expression was measured in colorectal cancers and cell lines. We discovered that an endogenous splice variant of TIA-1 encoding a truncated protein, short TIA-1 (sTIA-1) was expressed in CRC tissues and invasive K-Ras mutant colon cancer cells and tissues but not in adenoma cell lines. sTIA-1 was more highly expressed in CRC than in normal tissues and increased with tumour stage. Knockdown of sTIA-1 or over-expression of full length TIA-1 (flTIA-1) induced expression of the anti-angiogenic VEGF isoform $VEGF\text{-}A_{165}b$. Whereas flTIA-1 selectively bound $VEGF\text{-}A_{165}$ mRNA and increased translation of $VEGF\text{-}A_{165}b$, sTIA-1 prevented this binding. In nude mice, xenografted colon cancer cells over-expressing flTIA-1 formed smaller, less vascular tumours than those expressing sTIA-1, but flTIA-1 expression inhibited the effect of anti-VEGF antibodies. These results indicate that alternative splicing of an RNA binding protein can regulate isoform

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specific expression of VEGF providing an added layer of complexity to the angiogenic profile of colorectal cancer and their resistance to anti-angiogenic therapy.

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1. Introduction

Alternative RNA splicing, export and translational regulation all contribute to cancer formation and progression (Venables, 2006). These are dependent on the complex secondary structure of the 3′ UTR of the mRNA recognised by binding proteins such as T-cell Intracellular Antigen-1 (TIA-1) (Eberhardt et al., 2007). TIA-1 contains three domains with RNA recognition motifs (RRM1, RRM2 and RRM3), the latter two of which have been shown to be important for RNA binding and selectivity (Bauer et al., 2012) (Cruz-Gallardo et al., 2014). It has been shown to be alternatively spliced in exon 5 to form two isoforms (a and b, Supplementary Figure 1A) with b being the most active in its splicing activity, but TIA-1 also acts as a stress induced translational inhibitor, localising to stress granules with polyA RNA (Izquierdo and Valcarcel, 2007). TIA-1 alters both co-transcriptional and post-transcriptional RNA processing and binds VEGF-A RNA (Suswam et al., 2005), the alternative splicing of which influences the angiogenic capability of colorectal carcinomas (CRC) (Ferrara et al., 1991).

VEGF-A exists as multiple alternatively spliced isoforms. Exons 6 and 7 endow heparin binding (Houck et al., 1991), whereas exon 8 determines angiogenicity (Harper and Bates, 2008). Exon 8 has two splice acceptor sites, a proximal one, and a distal one, 66 bases downstream. The stop codon for the two isoforms lies the same number of bases downstream from the respective splice sites, resulting in two families of isoforms of the same amino acid number but with differing six amino acid sequence at the end, which alter the angiogenicity of the protein. Proximal splice site (PSS) selection in exon 8 results in the angiogenic family (VEGF-A_{xxx} where xxx is the number of amino acid e.g. VEGF-A_{165}), but distal splicing (DSS) results in VEGF-A_{xxx}b, anti-angiogenic proteins of the same size (e.g. VEGF-A_{165}b) (Bates et al., 2002). A variable but specific upregulation of the angiogenic isoforms is seen in colorectal cancer (Varey et al., 2008), but although the mechanisms underlying this splice specificity are being elucidated in epithelial cells (Amin et al., 2011; Nowak et al., 2008) and lung cancer (Merdzhanova et al., 2010), isoform specific control of expression, particularly in colon cancer is still not known. We therefore tested the hypothesis that TIA-1 could regulate VEGF splicing.

2. Materials and methods

2.1. Cells and tissues

HEK293, colonic carcinoma cell lines HT29, HCT116, HAC7, SW480, Osteosarcoma SaOS-2 and U2OS, the cervical cancer cell line, HeLa, prostate cancer cells, PC3 and LNCaP (ATCC; Rockville, MD) LS174t (ECACC, Salisbury, UK), adenoma cell lines AA/C1 and RG/C2, the *in vitro* transformed adenoma cell line AA/C1/SB/10, referred to as 10C cells) and breast cancer MDA-MB231 (Caliper, PerkinElmer, USA), Human Haemangioma Endothelial Cells (ESC and stem cells (HSC, a kind gift from Joyce Bischoff at Harvard University), myeloma RPMI-8226, and cardiac myocytes (HCM, from Promocell) were maintained as described previously (Williams et al., 1990) or according to manufacturer's instructions. Cancer cell lines were validated by STR profiling (Identicell, Aarhus, Denmark). Human tissues were collected under Local Ethics Committee Approval. An initial study was undertaken on six frozen paired colon samples (normal and cancerous) from partial colon resection. Additional RNA was extracted from 40 formalin fixed, paraffin embedded (FFPE) samples of normal and tumour tissues. These were taken from 22 male, 18 female patients, mean age 69 years, 28 with colon carcinoma, 18 with rectal carcinoma and 1 with carcinoma of the rectosigmoid junction.

2.2. Antibody generation

An antibody to sTIA-1 was generated by Abgent Europe using a KLH-conjugated VSLKNGQ NCPG peptide. For access please contact the corresponding author.

2.3. Protein analysis

Cell experiments used at least 3 independent biological replicates (independent experiments), with each western blot, ELISA and RT-PCR carried out on independently replicated samples. For western blot, and RT-PCR where gels are shown, each is indicative of a representative of 3 experiments, and quantitated as per the figures. ELISAs were undertaken on 3 separate sets of cells. Protein samples from homogenised tissue (100 μg) and whole cell extract (50 μg) were prepared using standard protocols. Protein lysates were resolved on SDS PAGE, and probed with a mouse monoclonal anti-COX-2 antibody (sc-19999; Santa Cruz), a/b-tubulin (Sigma) anti-VEGF-A_{165}b IgG (A56/1; R&D Systems), rabbit polyclonal anti-TIA-1 antibody (sc-28237; Santa Cruz, detects full length and sTIA-1 isoforms), or sc-166246 C10 mouse monoclonal N-terminal; Santa Cruz and rabbit anti-VEGF-A IgG (A-20, sc-120; Santa Cruz), goat polyclonal laminin (Santa Cruz) and mouse monoclonal lamin (Cell Signalling) antibodies. Proteins were detected using chemiluminescence and analysed by NIH image, or by fluorescently labelled secondary antibody imaging and imaged using a LiCOR Odyssey, and quantified using LiCOR imaging software. *ELISAs* were carried out as described previously (Varey et al., 2008). The VEGF-A_{165}b ELISA uses an

antibody that can detect all VEGF-A_{xxx}b isoforms but no VEGF-A_{xxx} isoforms, but its affinity to all of the different VEGF-A_{xxx}b isoforms is not known. We have assumed that it is the same as VEGF-A_{165}b and therefore calculated the levels making this assumption.

2.4. VEGF immunoprecipitation and mass spectrometry

For immunoprecipitation, 50 μl of protein A or protein G (depending on capture antibody) magnetic beads (Millipore) was washed with 500 μl PBS containing 0.1% Tween 20. After removing the buffer, the beads were resuspended in 100 μl of PBS/0.1% Tween, capture antibody added to the beads and incubated at room temperature for 10 min before adding the protein samples. The protein samples and antibody were incubated at 2–8 °C overnight with continuous mixing, washed with PBS/Tween0.1% before adding sample buffer. For spectrometry, after running the samples on PAGE, the gel was fixed in methanol:acetic acid (50%:7%) for 30 min and stained with SYPRO Ruby (Molecular Probes) overnight. The gel was de-stained in methanol:Acetic Acid (10%:7%), washed and imaged. The band was excised and subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides were fractionated using a Dionex Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) as described in the supplementary material. Raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the SwissProt Human database using the Mascot search engine (Matrix Science) with parameters as described in the supplementary material.

2.5. RNA analysis

Total RNAs from tissue (200 mg) and cells (10^7) was extracted using the RNeasy kit (Qiagen) and quantified. RNA was extracted from 25 μm thick sections of the FFPE samples by RNeasy FFPE kit (Qiagen) according to the manufacturer's protocol. Standard RT-PCR protocols were used. For cDNA synthesis form FFPE samples random primer or gene specific primers were used. Extracted DNA was cloned into the p-GEM T-Easy plasmid (Promega) and sequenced using standard synthetic primers complementary to the vector DNA at the junction of the cloning site (T7, SP6) and a pTarget sequencing primer 5′-TTACGCCAAGTTATTTAGGTGACA-3′ by the Dundee sequencing service (Dundee University). For each cell line 10 clones were sequenced.

RNA Immunoprecipitation RIP was performed as previously described (Cok et al., 2003; Tenenbaum et al., 2002). In brief, 5×10^7 cells were harvested, cross linked with 1%formaldehyde at room temperature for 10 min and quenched with 0.25 M glycine. Protein was extracted, and 100 μg of precleared protein incubated with 3 μg TIA-1 antibody immobilized using Seize Primary immunoprecipitation kit (Pierce Biotechnology) and immunoprecipitates isolated by centrifugation. Total RNA was extracted from immunoprecipitates and subjected to RT-PCR as described.

2.6. MS2-MBP binding experiments

The VEGF-A sequence from 35 bp upstream of exon 8a to 35 bp downstream of exon 8b was inserted into the ADML-MS2 plasmid as previously described (Nowak et al., 2008). Nuclear protein (75 μg) from HEK 293 or AA/C1/SB/10 (10C) cells were prepared and added to the MS2-MBP fusion protein/VEGF-RNA mix in 0.5 mM ATP, 6.4 mM $MgCl_2$, 20 mM creatine phosphate for 1 h at 30 °C. Proteins bound to the MS2-MBP/VEGF-MS2 RNA complex were affinity selected on amylose beads and immunoblot analysis was performed on 30 μg of protein samples using TIA-1 antibody and β-tubulin antibodies as previously described (Nowak et al., 2008). Blots are representative of at least three experiments.

2.7. TIA-1 construct and cell transfection

The cDNA for flTIA-1was generated by RT-PCR amplification using *Pfu* Turbo DNA polymerase (Stratagene) and primer TIA-1-forward and TIA-1-reverse from RNA from the RG/C2 adenoma cell line. The 1239 bp fragment was cloned in p-GEM T-easy vector and sequenced in both directions. The cDNA for both variants (13-exon TIA-1NCBI NM_022173.2 and 12-exon TIA-1NCBI NM_022037.2) were obtained using these primers. For *in vitro* expression, the cDNA were subcloned into mammalian expression vectors, pTarget (Promega) and pcDNA3 according to the manufacturer's instructions. siRNA to exon 6A was generated by Qiagene. Two custom siRNAs targeting sTIA-1 (siRNA1: CAGAATTGCCCTGGCTAACTA and siRNA2: GCCCTGGCTAA CTACAAGCTA) and control siRNA (Lopez de Silanes et al., 2005) were assessed. Two colon cancer cell lines were transfected with mixture of the 2 siRNAs (50 nM) using HiPerfect transfection reagents (Qiagen). Knockdown was monitored after 24 (RNA) and 48 (protein) hours. TIA-GFP plasmid is a kind gift from Paul Anderson (Harvard).

Transfection: LS174t and 10C cells were electroporated with 2 μg cDNA of pTarget-flTIA-1 cDNA in pTarget and pmaxGFP™ vectors pTarget vector or pmaxGFP™ with an AMAXA Nucleofector system using a basic epithelial cell transfection Kit (VPI-1005, Amaxa GmbH). Transfectants were analysed 24 h post nucleofection by Trypan blue staining and fluorescence microscopy for GFP control plasmid. Transfected cells were selected using G418 (500 μg/ml) for 4 weeks.

2.8. Immunofluorescence

The cells were 4% formaldehyde fixed (15 min) and incubated in 1%BSA/10% normal goat serum in TBS-Tween (0.1%) for 1 h. The cells were then incubated with the antibody (ab40693 rabbit polyclonal C-terminal, or sc-166246 C10 mouse monoclonal N-terminal, 5 μg/ml) overnight at +4 °C. The secondary antibody (red) was Alexa Fluor donkey anti-rabbit and (green) Alexa Fluor 488, used at a 1/500 dilution for 1 h. Vector shield Hoescht was used to stain the cell nuclei (blue). For confocal imaging a Nikon D-Eclipse C1 Laser Scanning Microscope was used. Cells were imaged using a 60× oil immersion objective with a numerical aperture (NA) of 1.4–5 cell clumps were selected at random and a z stack of 25 images taken for each

clump of cells. The central slice of each of these stacks was selected and used for the analysis of the ratio of staining between the nucleus (Hoescht) and cytoplasmic regions of the cell. In NIH image, the TIA-1 positive area and the Hoescht positive area were determined by tracing and nuclear area subtracted from the TIA-1 positive area and expressed as a proportion of the TIA-1 positive area.

2.9. *Luciferase reporters*

The VEGF-A full length 3′UTR was cloned downstream of the Thymidine Kinase promoter and the Firefly Luciferase ORF (construct LUC-VEGF$_{165}$), (from Jean-Jacques Feige and Nadia Cherradi, CEA Grenoble). This was modified using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) to delete the first 66 nucleotides (LUC-VEGF-A$_{165}$b) of exon 8. The plasmid pRL-TK (Promega) encoding Renilla Luciferase was used as an internal control. 10C cells were transfected with the both reporter constructs using the Amaxa system. Luciferase activities were measured with the DUAL-Luciferase reporter assay system (Promega) on a VICTOR 3 1420 Multilabel Counter Luminometer (PerkinElmer).

2.10. *Actinomycin experiments*

VEGF-A mRNA decay was measured in 10C cells after transfection with TIA-1 and treatment with 10 μmol/L actinomycin D. Cells were harvested every hour over a 4-h period, and VEGF-A isoform mRNA levels analysed by RT-PCR.

2.11. *Tumour growth in nude mice*

Experiments were undertaken under home office license after review by the University of Bristol Ethical review group. 2×10^6 untransfected, flTIA-1, VEGF-A$_{165}$, doubly or vector transfected cells in 0.2 ml PBS were injected subcutaneously in the dorsum of 6 Balb/C nude mice (from University of Bristol colony) for each group. Mice were treated bi weekly with mouse IgG (Sigma), or an anti-mouse VEGF-A neutralising antibody (Liang et al., 2006) intraperitoneally twice weekly at 2 mg/kg. Sample size was calculated a priori using G-Power on the basis of being able to have 80% power to detect a mean ± SD 2 ± 0.5 fold change in size and vascular density. All experiments were carried out with the investigator blinded to the groups.

2.12. *Immunohistochemistry*

5 μm thick de-waxed, re-hydrated sections were microwaved in 0.01 M sodium citrate, pH 6.0 for 15 min at 800 W. Sections were incubated with VEGF-A Receptor 2 (Rabbit Mab 2479 Cell Signalling) or CD31 antibodies at 1:100 overnight at 4 °C in a humidity chamber and after application of secondary antibodies and appropriate enzymatic process (Vector DAB kit), vessel number counted in 10 randomly selected areas for each tumour ($n = 6$).

2.13. *Statistical analysis*

Results were analysed using Prism software, given as Mean ± SEM unless otherwise stated and compared using one-way ANOVA or *t* tests for comparison of means. Proportions were compared using Fisher's exact test. $p < 0.05$ was considered significant. Power analysis on non-significant results were carried out to ensure power of at least 80% (using G-Power).

3. Results

3.1. *Ras mutant cancer cells express a novel splice variant of TIA-1*

In five carcinoma (LS174t, HCT116, SW480, HT29, HCA7) and an adenocarcinoma line (10C) TIA-1 RNA, but not two other RNA binding proteins, TTP and HuR, was lower than in adenoma lines RG/C2, and AA/C1 (Figure S1A, B), amplified using primers to detect full length (flTIA-1). Expression of the 42–43 kDa TIA-1 protein was similar across cell lines, but in all carcinoma and the 10C cells, an additional band at ~16 kDa was seen (Figure 1A, Figure S1C) suggesting the presence of a truncated TIA-1 protein. Amplification of TIA-1 mRNA sequence from the 10C cells using primers that flank exons 6 and 7 revealed an additional product only in carcinoma and 10C cells (Figure 1B, Figure S1D), due to the insertion of a 66bp sequence (Figure 1C) from the intron between exons 6 and 7 of the *tia-1* gene (Figure 1E). This was also present in BRAF V600E + ve A375 melanoma cells (Figure S1E). The 66base sequence is flanked by a classical consensus splice sequence (AG…GU) and has a 73% CU sequence immediately upstream, suggesting a cassette exon undergoing alternative splicing. This sequence results in a unique 11 amino acid sequence and a premature stop codon, which should generate a 144 amino acid truncated TIA-1 peptide of 15.8 kDa (Figure 1D), with only RNA recognition motif (RRM)-1 and part of the RRM 2, consistent with the short band (~16 kDa) detected by the anti-TIA-1 antibody in Figure 1A. Sequencing of TIA-1 genomic DNA from 10C cells showed no mutations in the region around either the 5′ or 3′ acceptor or donor sites. This mRNA is identical to a splicing event described as exon 6A when TIA-1 itself was over-expressed in a cancer cell line (Le Guiner et al., 2001), but its endogenous expression has not previously been demonstrated. A BLAST search of this sequence showed identity in 66 ESTs, all consistent with it being this TIA-1 isoform. A rabbit antibody raised to the unique peptide sequence of sTIA-1 detected the protein in 10C cells (Figure S1F). Immunoprecipitation of 10C protein (Figure S1G) and immunoblotting for TIA-1 showed both short and full length TIA-1. IP with a flTIA-1 antibody and IB with sTIA-1 demonstrated that the protein detected with this antibody was TIA-1 in LS174t cells as well. This protein was not present in AAC1 cells. (Figure S1H). Both protein (Figure 1E top image) and RNA expression (Figure 1E bottom image) of sTIA-1 was seen in sarcoma (SaOS), HeLa, prostate cancer (PC3 and LNCaP), and MDA-MB-231 breast cancer cells, but not osteosarcoma (U2OS), haemangioma endothelial (a differentiated phenotype), haemangioma stem (HSC), human embryonic kidney (HEK), or RPMI-8226 leukaemia cells or human cardiac myocytes

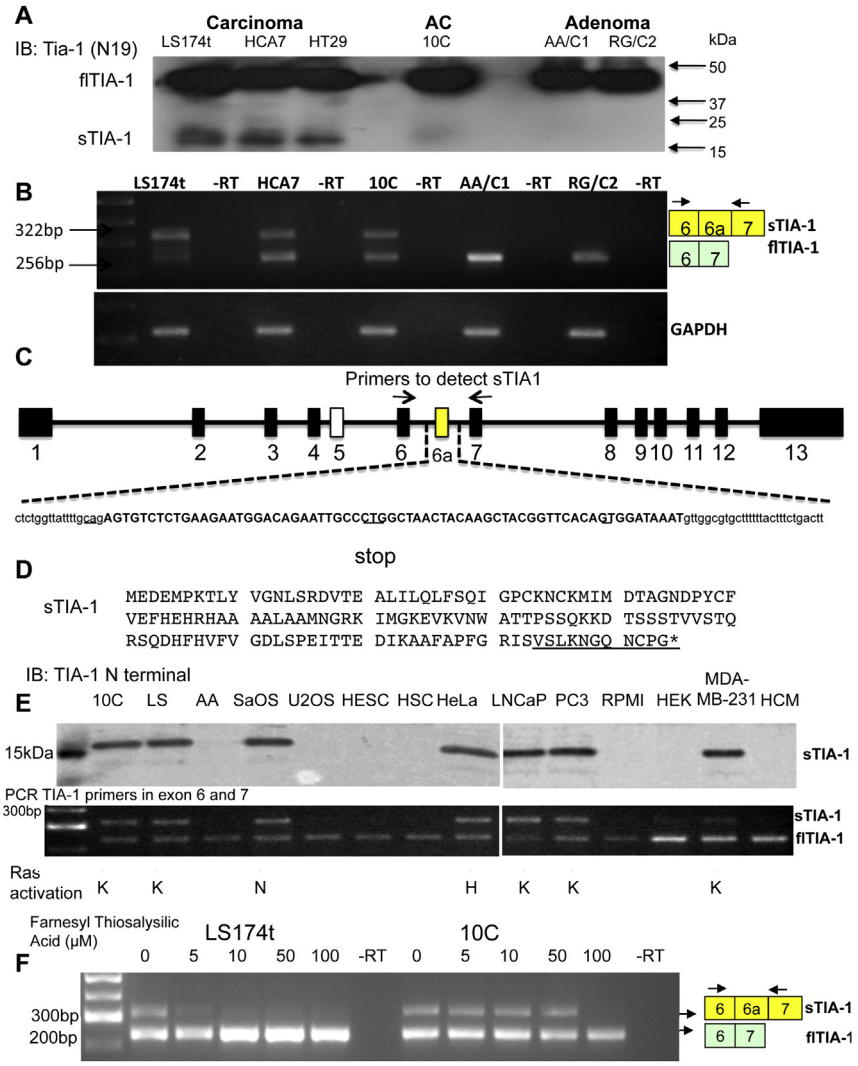

Figure 1 – TIA-1 splicing is modified in colon carcinoma cells. A. Immunoblot for TIA-1 in carcinoma, adenocarcinoma and adenoma cell lines. A smaller protein product (sTIA-1) was seen in carcinoma and adenocarcinoma (AC) cells. B. RT-PCR for TIA-1 using exon 6/7 spanning primers revealed two splice variants in LS174t, HCA7 and 10C cells. -RT = RNA without reverse transcriptase. C. Exon/intron structure, position of primers and sequence of exon 6a of TIA-1. D. Protein sequence of the translated mRNA product. Underlined section is unique to sTIA-1. E. Western blot and PCR for sTIA-1 of multiple cell lines. 10C-adenocarcinoma, LS174t colon carcinoma, AA – adenoma, SaOS-sarcoma osteogenic2, U2OS – osteosarcoma, HEC – haemangioma endothelial cells, HSC – haemangioma stem cells, HeLa – ovarian carcinoma, LNCaP – prostate cancer lymph node metastasis, PC3 prostate cancer, RPMI – RPMI8266 myeloma cells, MDA-MB-231 breast cancer cells, HCM-human cardiac myocytes. F. LS174t (K-ras activating mutation heterozygous) and 10C (K-ras homozygous) were treated with increasing concentrations of the Ras inhibitor FTA and subjected to RT-PCR for TIA-1. Ras inhibition prevented splicing of sTIA-1.

(HCM). Of the 18 cell lines in which sTIA-1 was investigated, only 2 of the 8 that lacked sTIA-1 have ras mutations or over-expression, whereas 8 of the 10 that did express sTIA-1 had ras over-expression or activating mutations, and the other two are BRAF mutants, (HT29 and A375 cells). In both LS174t and 10C cells the Ras inhibitor Farnesyl Thiosalysilic Acid (FTA) significantly inhibited splicing to sTIA-1 in a dose dependent manner (Figure 1F). Downstream signalling through MEK and PI3K was required for this splicing in both cell lines (Figure S2A). Ras is active in AAC1 and RGC2 cell lines (sTIA-1 negative) indicating that it is necessary, but not sufficient to induce splicing of TIA-1.

3.2. *TIA-1 variants differentially regulate VEGF isoform expression*

Ras inhibition (Figure 2A, Figure S2B–D) and MEK and PI3K inhibition (Figure S2E) also switched splice form expression from VEGF-A_{165}a to VEGF-A_{165}b in both 10C and LS174t cell types. VEGF-A_{165}b protein expression was confirmed by immunoprecipitation and peptide sequencing (Figure S3A and B). These results suggest that hyperactivated ras signalling also acts to repress VEGF-A_{xxx}b splicing, so we investigated a possible link between VEGF and TIA-1. Isoform specific siRNA knockdown of sTIA-1 (Figure S3C–G), reduced

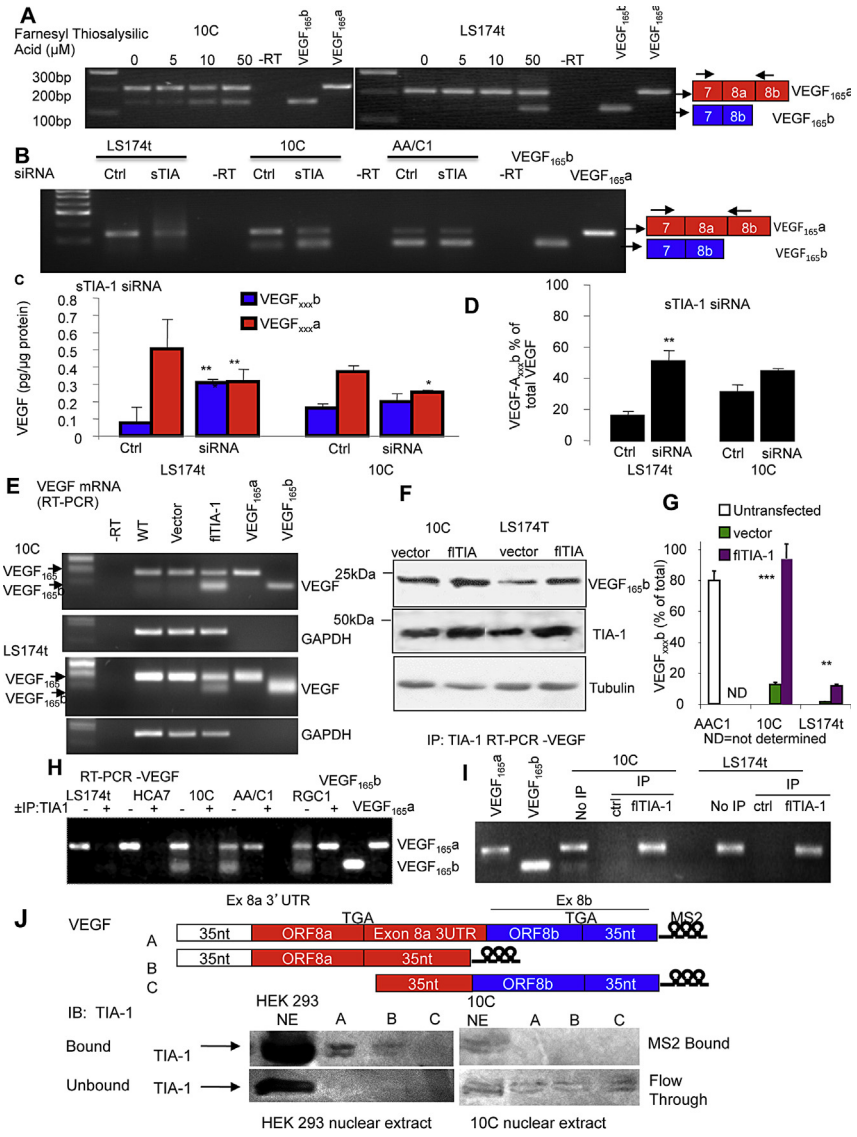

Figure 2 – TIA-1 regulates VEGF splice form expression. A. PCR for VEGF-A showing ras inhibition by FTA increased VEGF-A_{165}b expression. B. Colonic tumour cells were transfected with 2 siRNAs targeted to exon 6A of TIA-1 (sTIA), or scrambled siRNAs (Ctrl), and RNA extracted, reverse transcribed and subjected to PCR for VEGF-A isoforms. Plasmids containing VEGF-A_{165}a or VEGF-A_{165}b were used as positive controls. C. Protein was extracted from such transfected cells, quantitated and subjected to ELISA for VEGF-A_{xxx}b and total VEGF. VEGF-A_{xxx}a levels were calculated from the difference between total and VEGF-A_{xxx}b. D. Percent of total VEGF-A that is VEGF-A_{xxx}b found in LS174t or 10C cells after siRNA knockdown of sTIA-1, measured by ELISA. E. Untransfected cells, or cells transfected with pcDNA3 control (vector) or flTIA-1 expression vector had mRNA extracted and were subjected to RT-PCR using primers that detect both proximal (PSS, VEGF-A_{165}a) and distal spliced isoforms (DSS, VEGF-A_{165}b). F. Protein extracted from cells treated in this way was subjected to SDS PAGE and immunoblotted with an antibody to VEGF-A_{165}b, TIA-1 or tubulin (loading control). G. Protein was subjected to ELISA for pan-VEGF-A and VEGF-A_{165}b. The percent of total VEGF-A that is VEGF-A_{165}b is shown. ND- = Not determined. See also Figure S2. H. Cells were lysed, and part of the lysate cross linked, and precipitated with an antibody to TIA-1 (+), and the remainder (−) subjected to RNA extraction. After IP the RNA was extracted from the precipitate and both RNA samples subjected to reverse transcription and PCR for VEGF-A isoforms. Adenoma but not carcinoma cell VEGF-A_{165} RNA was precipitated by TIA-1 antibody. I. flTIA-1 transfected and untransfected colon tumour (10C or LS174t) cells were crosslinked, immunoprecipitated with TIA-1 antibody, RNA extracted and subjected to RT-PCR for VEGF. cDNA from cells without immunoprecipitation (no IP) was also subjected to RT-PCR for VEGF. Lack of binding of VEGF-A_{165} RNA to TIA-1 in sTIA-1 expressing cells was rescued by flTIA-1 over-expression. J, Constructs *in vitro* transcribed and used to investigate mRNA binding of TIA-1. Nuclear extract (NE) of HEK293 cells (left) or 10C (right) was incubated with *in vitro* transcribed mRNA constructs A, B or C, and run over an MS2-MBP binding column. Bound protein, and unbound (flow through) was run on SDS PAGE and immunoblotted for TIA-1. ORF = Open Reading Frame. ** = $p < 0.01$, * = $p < 0.05$ compared with ctrl. One-way ANOVA. Post hoc Bonferroni test.

VEGF-A_{165}a mRNA in both 10C and LS174t and increased VEGF-A_{165}b mRNA in 10C cells but made no difference in AA/C1 cells (Figure 2B). VEGF-A_{165}b but not VEGF-A_{165} protein was significantly increased by siRNA to sTIA-1 in LS174t cells ($p = 0.013$), whereas only a significant reduction in the VEGF-A_{165}a isoform expression was seen in 10C cells ($p = 0.03$, Figure 2C). Thus there was a significant switch in the ratio of isoforms in both cell types towards a less angiogenic phenotype after knockdown of sTIA-1 (Figure 2D, $p < 0.01$ ANOVA). Conversely, VEGF-A_{165}b expression was rescued by flTIA-1 expression in 10C and LS174t cells measured by RT-PCR (Figure 3E), immunoblot (Figure 2F) and ELISA (Figure 2G), in 10C cells to 90% VEGF-A_{165}b ($p < 0.001$ compared with ctrl), similar to AA/C1 cells and in LS174t from 5 ± 1.8% to 12 ± 1.5% of total VEGF-A ($p < 0.01$). The reverse experiment (over-expression of sTIA-1) resulted in lethality of unknown cause. flTIA-1 also reduced cyclooxygenase-2 protein expression (Figure S3H), a known

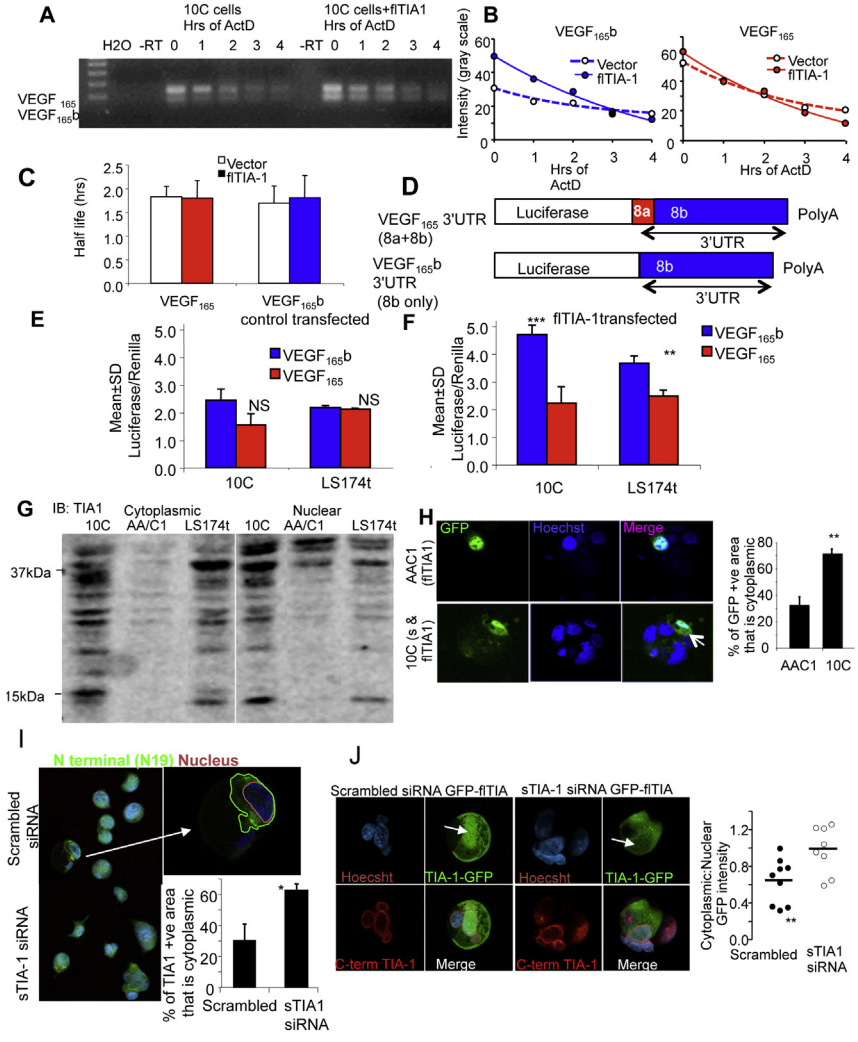

Figure 3 – TIA-1 localisation regulates VEGF-A mRNA translation but not degradation. A. 10C cells, or 10C cells expressing flTIA-1 were treated with the transcriptional inhibitor Actinomycin D (ActD) followed by RT-PCR for VEGF. B. The intensity of the PCR product was measured with image analysis and the degradation rate determined by exponential curve fit (bottom graphs). C. Half-life of VEGF-A_{165}a and VEGF-A_{165}b mRNA in the presence or absence of flTIA-1. D. VEGF-A 3′UTR constructs were generated downstream of firefly luciferase in a Luciferase/Renilla reporter gene This was then transfected into 10C and LS174t in the absence (E) or presence (F) of flTIA-1 over-expression. Luciferase and Renilla were measured in these cells. *** = $p < 0.001$, ** = $p < 0.01$. NS = Not significant Repression of VEGF-A_{165} relative to VEGF-A_{165}b was seen when flTIA-1 was transfected. G Protein was extracted from AAC1 (K-ras wild type) and 10C and LS174t cells (both K-ras mutant), fractionated into nuclear and cytoplasmic fractions, and subjected to immunoblotting. TIA-1 expression was found predominantly in the nucleus in AAC1 cells, but in both nucleus and cytoplasm in sTIA-1 expressing cells. H. AAC1 (endogenously express flTIA-1) and 10C cells (endogenously express both sTIA-1 and flTIA-1) were transfected with TIA-1-GFP construct. Nuclei were stained with Hoescht and cells were imaged by confocal microscopy. The proportion of GFP outside the Hoescht staining (arrow) was calculated from the area of staining outside the nucleus as a proportion of the total staining area from a single confocal slice through the nucleus (slice). I. Cells were transfected with siRNA to sTIA-1 or scrambled siRNA, and stained for endogenous TIA-1 with an antibody to the N terminus. The proportion of TIA-1 that as cytoplasmic was calculated. * = $p < 0.05$ compared with scrambled. J. 10C cells were transfected with scrambled or sTIA-1 siRNA and flTIA-GFP fusion protein. The intensity of the expression in the cytoplasm and the nucleus was determined. ** = $p < 0.01$ compared with scrambled unpaired *t* test.

TIA-1 target. RNA immunoprecipitation (RIP) of TIA-1 showed that, whereas 10C, AA/C1 and RG/C1 all expressed both VEGF-A_{165} and VEGF-A_{165}b, immunoprecipitation with an anti-TIA-1 antibody pulled down only VEGF-A_{165} in AA/C1 and RG/C1 (cells expressing only flTIA-1), but no VEGF-A mRNA in sTIA-1 expressing cell lines (10C, HCA7 and LS174t, Figure 2H). In 10C and LS174t cells, transfection with flTIA-1 restored TIA-1 binding to VEGF-A_{165}a mRNA, as evidenced by pull down of VEGF-A_{165} mRNA, compared with untransfected cells (ctrl, Figure 2I). sTIA-1 also affected binding of TIA-1 to COX-2 (Figure S3I). The binding site of TIA-1 to VEGF-A mRNA was demonstrated, in HEK cell nuclear extract using an MS2-MBP binding assay (Nowak et al., 2008) to reside in the VEGF-A exon 8a sequence and 35 bases of intron 7 (Figure 2J). Nuclear extract from 10C cells, while containing TIA-1 (as seen in the flow through unbound eluate in the lower panel), was unable to bind to VEGF-A mRNA sequences (Figure 2J).

flTIA-1 has been shown to function both as a translational repressor (Piecyk et al., 2000) and an alternative splicing factor (Barron and Lou, 2012; Del Gatto-Konczak et al., 2000). Expression of flTIA-1 did not affect the half-lives of VEGF-A_{165}b and VEGF-A_{165} mRNA in 10C cells, determined by investigating mRNA decay rates in cells treated with the transcriptional inhibitor Actinomycin D (ActD) (Figure 3A and B). Although it did increase the amount of VEGF-A_{165}b at the start of the experiment, Figure 3C shows that there was no difference in the half-life. There was no significant difference in expression of a luciferase reporter containing the 3′UTRs of VEGF-A_{165}a or VEGF-A_{165}b (Figure 3D) in either 10C or LS174t cells (Figure 3E), but transfection with flTIA-1 resulted in significantly increased luciferase activity with the VEGF-A_{165}b 3′UTR compared with the VEGF-A_{165}a 3′UTR (Figure 3F) indicating preferential translation of VEGF-A_{165}b by TIA-1 as well as inhibiting splicing of VEGF-A_{165}. The flTIA-1 isoform contains three RNA recognition motifs (RRMs). RRM 2 and 3 are required for 3′ translational repression (Bauer et al., 2012), and this is lacking in sTIA-1, which only contains the full length RRM1, which cannot by itself bind to RNA (Dember et al., 1996) but is required for flTIA-1 binding to specific sequences (e.g. U rich sequences) (Bauer et al., 2012), thus conferring specificity. Thus, while sTIA-1 prevents flTIA-1 mediated repression of splicing of the pro-angiogenic isoforms of VEGF-A, the increased level of VEGF-A_{165}b mRNA and protein induced by over-expression of flTIA-1 in colon cells suggest that it also acts to selectively represses translation of the exon 8a containing isoforms. This may explain why the effects of sTIA-1 siRNA knockdown on RNA and protein were subtly different in the two cell types (in 10C it increased VEGF-A_{165}b RNA but not protein, and vice versa in LS174t cells).

3.3. *sTIA-1 alters nuclear localisation of flTIA-1*

Nuclear/cytoplasmic separation of proteins (Figure S4A) showed that in AAC1 cells, TIA-1 is found as the full length form but only in the nucleus, whereas in LS174t and 10C cells a substantial proportion of all TIA-1 isoforms and products were expressed in the cytoplasm, including sTIA-1 (Figure 3G). This was confirmed by transfection of cells with full length TIA-1 fused to GFP (Figure 3H), and by immunofluorescent staining with antibodies to the N terminus of TIA-1 (detects all isoforms, Figure S4B). sTIA-1 knockdown, surprisingly, resulted in *enhanced* cytoplasmic staining for TIA-1 (Figure 3I). This was confirmed in flTIA-1-GFP over-expressing 10C cells, with sTIA-1 knockdown (Figure S4C). N-terminal staining (which would detect sTIA-1) was reduced, consistent with a reduction in only the sTIA-1 component (Figure S4C). Nuclear localisation of GFP-flTIA-1 was reduced by sTIA-1 knockdown (Figure 3J). sTIA-1 therefore alters localisation of the flTIA-1 within the cell, thereby preventing it from binding VEGF-A mRNA. The increased not decreased cytoplasmic TIA-1 resulting from reduced of sTIA-1 in K-Ras mutant cells suggests that the GFP-flTIA-1 fusion protein localisation is sTIA-1 dependent. Thus sTIA-1 expression in K-Ras mutant cells may act by preventing flTIA-1 nuclear trafficking (to and from the nucleus), preventing it from interacting with other potential regulators of splicing and/or translation. This in turn alters the expression of VEGF-A isoforms and results in a more angiogenic phenotype of the tumours. Co-immunoprecipitation indicated that it is possible that the two isoforms could complex together (Figure S1G), but how this happens is not clear. The localisation experiments indicate that in carcinoma cells flTIA-1 excess either by over-expression with GFP, by knockdown of sTIA-1 or both results in cytoplasmic flTIA-1 and/or VEGF-A_{165}b expression, whereas in adenoma cells, where K-Ras is not over-expressed, flTIA-1 is nuclear. Thus when sTIA-1 dominates in the nucleus, VEGF-A_{165} is generated.

3.4. *sTIA-1 is expressed in human K-Ras mutant colorectal tumours*

sTIA-1 and VEGF-A_{165}b RNA were detected in freshly isolated human colorectal cancer samples (Figure 4A) confirmed by sequencing. Quantification showed both a significant increase in sTIA-1 expression in cancer (Figure 4B) and a significantly lower VEGF-A_{165}b:VEGF-A_{165} ratio in the tumours than the controls (Figure 4C). In histological samples, flTIA-1 RNA (Figure 4D) was found only in 13 of 31 tumours (42%) but 22 of 30 normal tissues (73%, $p = 0.02$, Fisher's exact test) samples (Figure 4E). The proportion of tumours that were K-Ras mutant was significantly higher ($p < 0.01$) in those that had no flTIA-1, and K-Ras mutant tumours rarely (2/13 cases) had flTIA-1 detected (Figure 4F). Quantification showed that sTIA-1 exceeded flTIA-1 in 20 of 31 tumours (65%) but in only 9 of 30 normal tissues (30%, $p = 0.01$, Figure 4G). Moreover, sTIA-1 was present in 14 of the 15 Dukes' C (93%), but only 9 of the 16 Dukes' B (56%, $p < 0.01$, Figure 4H). There was a significantly higher microvascular density (Figure 4I) in samples in which sTIA-1 but not flTIA-1 was found (Figure 4J).

3.5. *flTIA-1 over-expression reduces tumour growth and results in bevacizumab resistance*

Over-expression of flTIA-1 inhibited tumour growth in nude mice (Figure 5A), resulting in significantly smaller tumours at 18 and 21 days after injection (Figure 5B). This reduction was rescued by VEGF-A_{165} cDNA expression i.e. VEGF-A that was not under the control of alternative splicing (Figure 5B). Tumours from flTIA-1 transfected cells had reduced tumour

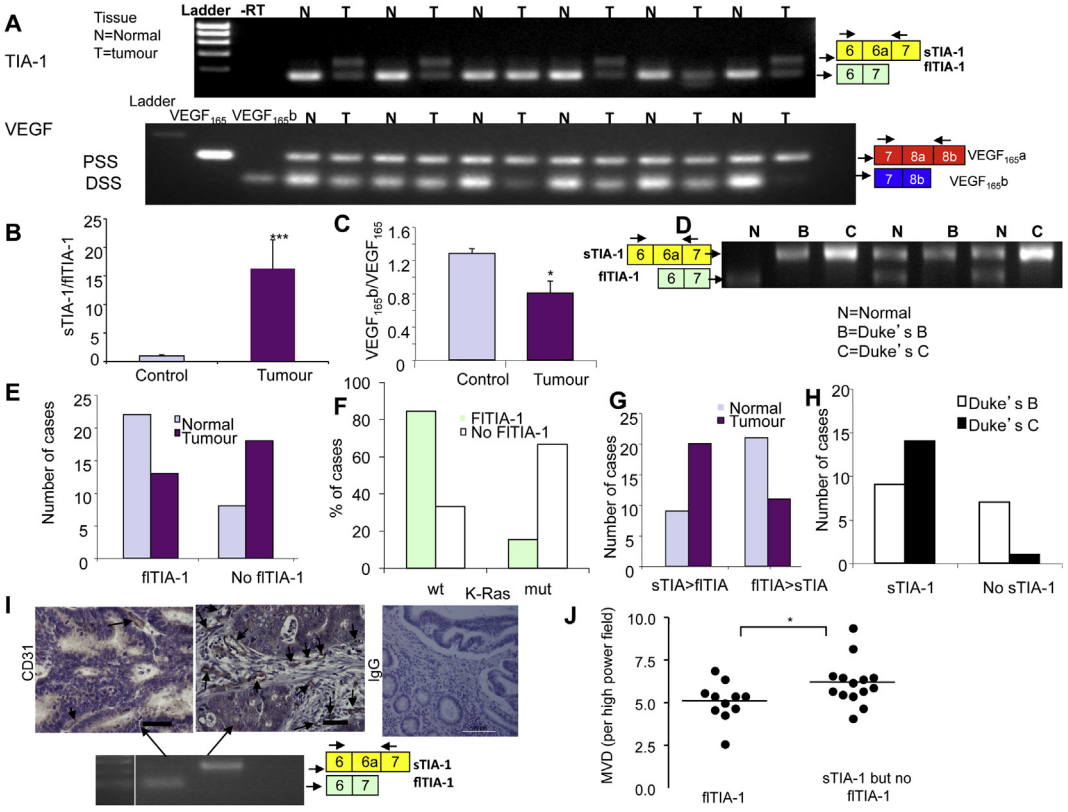

Figure 4 – sTIA-1 splice variants are present in human colon cancer. A. Upper panel, RT-PCR of mRNA extracted from paired tumour (T) and normal (N) tissues from 6 patients undergoing bowel resection for colon cancer. In 4 of 6 patients, the splice isoform were present in the tumours, but not in the normal tissue. Lower panel, RT-PCR for splice forms of VEGF. B. Quantitation of band intensity of sTIA-1 relative to flTIA-1 in control and tumour tissue. C. Quantitation of band intensity of VEGF-A_{165}b relative to VEGF-A_{165} in colon cancers compared with normal tissues. D. RNA was extracted from 25 μm thick scrolls of paraffin embedded sections of 40 colorectal carcinoma (20 Duke's B, 20 Duke's C stage) and matched normal (N) samples, and RT-PCR carried out to detect flTIA-1 and sTIA-1. RT-PCR detected TIA-1 RNA expression in 61/80 samples. E. Number of cases in which flTIA-1 was found in tumour or normal ($p = 0.02$ Fishers exact test). F. Number of cases in which flTIA-1 was found (black bars) or not found (white bars) in wildtype versus ras mutant tumours. G. Number of cases in which sTIA-1 was more highly expressed than flTIA-1 (compared with normal $p = 0.0106$). G Number of cases in which sTIA-1 was detectable in Duke's B compared with Duke's C grade ($p = 0.018$, statistics for E, F and G Fisher's exact test). H. Sections adjacent to those from which RNA had been extracted were stained for blood vessels (CD31). Blood vessels were counted per high power field, blinded for sTIA-1 status. The gel shows the PCR product for the two sections shown. Arrows indicate blood vessels. I. Microvascular density for sections where flTIA-1 was more intense than sTIA-1 and vice versa. * = $p < 0.05$ unpaired t test.

vessel density (Figure 5C and D, $p < 0.01$). Mice with LS174t tumours expressing flTIA-1 treated with an anti-VEGF-A antibody (Liang et al., 2006) grew significantly faster compared with IgG (Figure 5E), at the same rate as wild type tumours treated with anti-VEGF-A treatment (Figure 5F), but still more slowly than untreated wild type tumours (Figure 5G).

4. Discussion

Here we identify a novel K-ras dependent endogenous splice variant of TIA-1, short TIA-1 (sTIA-1) in CRC patients and cancer cells, which controls VEGF-A isoform expression. This sTIA-1 had been identified as being capable of being induced by over-expression of recombinant mouse TIA-1 in human HEK cells (Le Guiner et al., 2001). This is the first demonstration that this splice variant may be present in human cancers, and that it may regulate VEGF splice form expression. sTIA-1, containing only one RNA recognition motif (RRM), prevented binding of full length TIA-1 to VEGF-A_{165} mRNA, blocking both its angiogenic VEGF splice site repression and its promotion of translation of anti-angiogenic VEGF-A_{165}b. It has previously been shown that RRM2 and 3 are required for binding of TIA-1 to RNA (Bauer et al., 2012), and interestingly, RRM3 is required for binding to C rich stretches (Cruz-Gallardo et al., 2014). Moreover, inclusion of exon 5 in TIA-1 results in a higher splice activity, indicating that the activity of the protein can be altered by alternative splicing (Izquierdo and Valcarcel, 2007). As both of these domains are disrupted in sTIA, it is likely that sTIA-1 does not act by directly binding to the VEGF RNA and preventing flTIA-1 from exerting its effects, and the direct mechanism of repression of distal splicing is not known. As sTIA-1 did co-immunoprecipitate with flTIA-1 it is possible that sTIA-1 acts by heterodimerising with flTIA-1 and preventing it from repressing splicing and/or translation of the pro-angiogenic isoform, VEGF-A_{165}a. Cancer cells over-

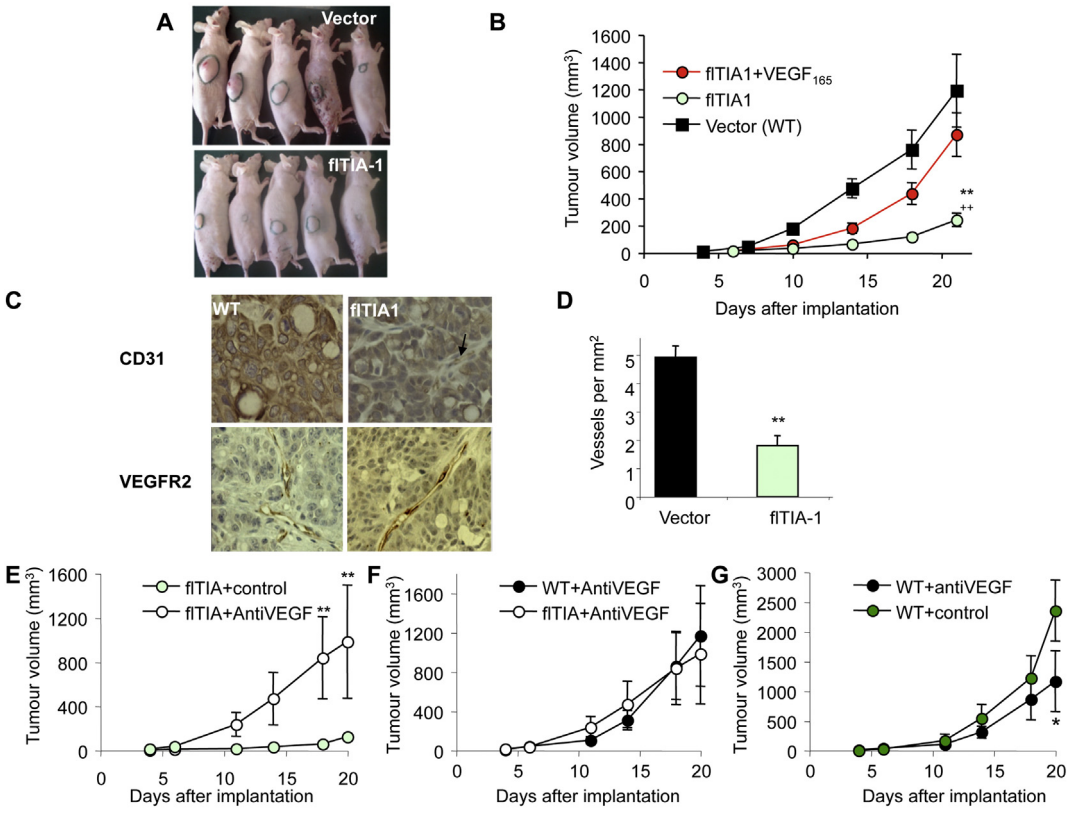

Figure 5 – flTIA-1 over-expression reduces growth of sTIA-1 expressing tumours *in vivo* in a VEGF dependent manner A. 2×10^6 control transfected (vector), flTIA-1 transfected cells or double VEGF-A_{165}a and flTIA-1 transfected LS174t colon carcinoma cells were injected into 6 nude mice per group and tumour volumes measured by callipers. A. Tumours grown in nude mice. B. Mean ± SEM tumour volumes calculated from the diameters. C. Tumours were stained with CD31 and VEGFR2 and vessels counted under 40× objective ($n = 6$). D. Tumour vessel densities. * = $p < 0.05$, ** = $p < 0.01$ compared with vector. E. LS174t cells expressing flTIA-1 were implanted into mice, and the mice treated with either 2 mg/kg IgG (control) or 2 mg/kg anti-VEGF-A antibody bi weekly. $p < 0.01$ two way ANOVA. F. Vector transfected (wild type, WT) or TIA-1 expressing cells were implanted into mice and treated with 2 mg/kg Anti-VEGF-A antibody twice per week. G. WT LS174t cells were treated with control or anti-VEGF-A antibody biweekly. Tumour volumes were measured by calliper. $P < 0.05$ two way ANOVA. ** = $p < 0.01$, * = $p < 0.05$ compared with control, Bonferroni post hoc test.

expressing flTIA-1 formed smaller, less vascular tumours than those expressing sTIA-1, and were more resistant to anti-VEGF antibodies such as bevacizumab. These results indicate that K-ras mediated splicing of an RNA binding protein may regulate isoform specific expression of VEGF, providing an added layer of complexity to the angiogenic profile of colorectal cancer and their resistance to anti-angiogenic therapy. This opens up the question as to what is the downstream mechanism through which K-Ras regulates TIA-1 splicing. There is no clear evidence as to how K-Ras activation can induce splicing switches. Splicing is regulated by the complex interaction of splicing factor proteins that can be phosphorylated, localise to the nucleus and take part in a complicated interaction with other RNA binding proteins and auxillary factors that control splice site location. The mechanisms through which TIA-1 is spliced is not known, and none of the known splicing factors have yet been identified as controlling Exon 6A splicing. Interestingly, over-expression of human flTIA-1 did not induce sTIA-1 splicing in cancer cells, in contrast to that shown by mouse flYIA-1 in human HEK cells (Le Guiner et al., 2001).

The finding that over-expression of VEGF-A_{165} can reverse the effect of flTIA-1 expression on tumour growth inhibition suggests that the effect was VEGF splicing dependent, through anti-angiogenic activity, or through direct effects on the cells. These findings may have significant therapeutic implications, but are not directly translatable. Low VEGF-A_{165}b levels predict bevacizumab response in CRC (Bates et al., 2012), but, bevacizumab provides significant clinical benefit in patients irrespective of K-ras mutations (Hurwitz et al., 2009). Although patients with K-ras mutations did do better on bevacizumab compared with controls (9.3 compared with 5.5 months, an increase of 3.8 months), they did not perform as well as patients with wild type K-ras (13.5 versus 7.4, an increase of 6.1 months). It would therefore be interesting to know how patients with K-ras mutations, *in which sTIA-1 is expressed*, responded. It is not yet clear whether sTIA-1 predicts survival, or can drive angiogenesis by reducing the anti-angiogenic VEGF-A_{165}b splice variants, or by increasing the VEGF-A_{165} variant. These VEGF-A_{165}b variants have been shown to be down regulated so far in melanoma (Pritchard-Jones et al., 2007), colon (Varey et al., 2008), prostate (Woolard et al.,

2004), and kidney (Bates et al., 2002) cancers, but the mechanism of regulation of these variants in cancer is still poorly described (Amin et al., 2011; Nowak et al., 2010, 2008). The loose relationship between sTIA-1 and VEGF-A_{165}b down regulation in patients suggests a complex interaction, just as for alternative splicing of p73 and VEGF-A_{165}b isoforms, also demonstrated in colon cancer (Diaz et al., 2008). This is supported by the findings that Ras inhibition in 10C cells was more effective at lower doses than in LS174t cells, and that the RNA and protein levels of VEGF-A_{165}b in the presence or absence of shTIA-1 are not directly correlated. The effect of TIA-1 on RNA stability, as shown in Figure 3 indicates that TIA-1 is likely to act in a pleiotropic manner on the amount of VEGF isoforms present – acting by altering translation specifically for one set of isoforms, by regulating splicing at the proximal splice site, or, most likely, by changing both splicing and translation. Binding of TIA-1 to the VEGF RNA was shown, but it is also possible that other vascular growth factors are regulated by TIA-1, although these have not yet been published.

In summary, we show that alternative splicing of TIA-1 in human colon cancer may regulate the balance of the pro- and anti-angiogenic VEGF-A isoforms, and control tumour growth and angiogenesis in animal models. This suggests that TIA-1 can act as a splicing and translational regulator of VEGF-A and alternative splicing of TIA-1 may lead to enhanced angiogenesis and tumour growth in colorectal cancer, and resistance to anti-VEGF-A therapy.

Contributions

MHZ, EMA, CHA designed and carried out experimental procedures, ED, KS, XY, KH, AS, OD'S, KBB carried out experimental procedures, ACW, DJK, AHJS, RSM, MRL, and SJH contributed to the design and secured funding for the experimental work, AHRV contributed to design and interpretation of the experiments, DOB designed the experiments, secured funding, and wrote the paper. All authors contributed to the writing of the manuscript and revised the draft paper.

Conflict of interest

DOB and SJH are holders of a patent on VEGF-A_{165}b.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.molonc.2014.07.017.

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