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ETV4 and Myeov knockdown impairs colon cancer cell line proliferation and invasion

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Abstract

We have identified novel colorectal cancer-associated genes using NCBI's UNIGENE cDNA libraries. Colon cancer libraries were examined using Digital Differential Display and disease-associated genes were selected. Among these were ETV4 and MYEOV, novel colorectal cancer-associated genes. Samples of matched normal and neoplastic colon were obtained from human subjects and gene expression was quantified using real-time PCR. ETV4 gene expression was significantly increased in colonic neoplasia in comparison to matched normal colonic tissue ($p < 0.05$). Myeov expression was also increased in colon neoplasia in comparison to matched normal tissue. The effect of siRNA-mediated knockdown of ETV4 and Myeov on cell proliferation and invasion was assessed. ETV4 knockdown resulted in a 90% decrease in cell proliferation ($p < 0.05$) and a 67% decrease in cell invasion. Myeov knockdown resulted in a 48% decrease in cell proliferation ($p < 0.05$) and a 36% decrease in cell invasion. These data suggest that ETV4 and Myeov may provide novel targets for therapeutic intervention.

Keywords: Colon cancer; Digital Differential Display; ETV4; Myeov; Cell proliferation; Cell invasion

Colorectal cancer is the second most common cause of cancer mortality and accounts for 10% of all cancers diagnosed [1,2]. Approximately half of all western populations will develop a precursor lesion, an adenoma, or carcinoma by age 70. The molecular portraits of those individuals most likely to progress from polyps to cancer, those most likely to have aggressive cancer, and those most likely to respond to adjuvant therapy remain poorly defined.

cDNA libraries derived from disease tissue provide a comprehensive means of studying tumour biology in colorectal cancer. High-throughput DNA sequencing initiatives have led to the generation of vast amounts of sequence data from diseased tissues, and their accumulation in public repositories [3]. Digital Differential Display (DDD) is a web-based resource (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) that facilitates the comparison of cDNA abundance in selected tissue libraries, providing a quantitative measure of gene expression. In this way, the transcriptomic signature of a given tissue type can be determined and novel disease-associated genes identified [4]. Digital Extractor, a program for the high-throughput processing of datasets derived from DDD-based comparisons of EST libraries, was previously developed by our group

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[5]. We have recently used this approach to identify genes of functional importance in gastric adenocarcinoma [6].

DDD is a powerful tool in delineating the molecular mechanisms underpinning complex diseases such as cancer. However, the functional significance of cancer-associated genes identified by DDD is often unclear. Appreciating this, we sought to examine the output of cDNA library profiling using a functional approach.

Materials and methods

Digital differential display. We utilised the cDNA library screening strategy, *Digital Differential Display*, to examine human tissue libraries at the NCBI's UniGene suppository and identify genes with enhanced expression in colorectal cancer. At the time of data-mining, the UniGene sequence repository contained 15 EST libraries derived from colorectal cancers only (Table 1). These libraries contained over 80,000 DNA sequences for use in gene mining. As a control, we compared the colorectal cancer libraries to normal colon libraries and normal adult tissue libraries. There were 110 libraries derived from normal human adult tissue, containing over 600,000 sequences, and one library derived from normal colon (5000 sequences).

Tissue collection and cell culture. Samples of normal colonic mucosa and matched sporadic adenomatous polyps and carcinomas were removed at colonoscopy and immediately stored in an RNAse inhibitor (RNAlater, Ambion, Austin, TX, USA) at −20 °C (as per manufacturer's recommendations). The number of tissue samples studied was three normal, six adenomatous polyps, and three carcinomas (Table 2). Tissue was retained with informed patient consent according to a protocol approved by the local Ethics Committee. Age and sex of the donors and tumour location and size were noted. All normal and neoplastic samples were reviewed by a histologist and described according to morphology and degree of dysplasia. An in vitro model of colon carcinoma was established using the T84 colon cancer cell line. The cell lines were cultured in Dulbecco's modified Eagle's medium-F12, with 1 U/ml penicillin, 1 μg/ml streptomycin, and 10% foetal bovine serum under standard conditions.

RNA preparation and PCR. TRIzol™ (Sigma–Aldrich, Ireland) was used to extract RNA from tissue samples (as per manufacturer's recommendations). Tissue disruption was facilitated using a Polytron 2100 PT rotor/stator homogeniser (Kinematica Inc., USA). Reverse transcription was achieved using AMV reverse transcriptase (Invitrogen Ltd., UK). Real-time RT-PCR was performed using a TaqMan ABI 7700 Sequence Detection System® (Applied Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems, Weiterstadt, Germany). GAPDH, which was amplified in parallel with the genes of interest, served as a housekeeping gene. Controls consisting of dH_2O were negative in all runs. All measurements were performed in triplicate. The oligonucleotide primers and probes employed in this study were: MYEOV, forward probe (FP): 5′-CCT AAA TCC AGC CAC GTC AT-3′, reverse probe (RP); 5′-GAC ACA CCA CGG AGA CAA TG-3′, FAM: 5′-GTG GGA TCT GAG CAG AGT CCA TTC A-3′; ETV4, (FP): 5′-CCG GTT TGT CAG TTC TTG GT-3′, (RP) 5′-AGA TGT GGT GGA GGT GGA AG-3′; FAM: 5′-TCG CCT ACG ACT CAG ATG TCA CCG G-3′; GAPDH (FP): 5′-GAAGGTG AAGGTCGGAGTC-3′, (RP) 5′-GAAGATGGTGATGGGATTTC-3′; FAM: CAAGCTTCCCGTTCTCAGCC.

siRNA knockdown. The functional roles of selected genes were assessed using gene knockdown with small interfering RNA (siRNA). siRNA was

Table 1
Colon cancer DDD libraries used for comparisons

dbEST ID	Library name	Origin	Sequences
5605	NIH_MGC_65	Colon cell line	7451
1540	NCI_CGAP_Co16	Bulk adenocarcinoma	8783
840	Colon carcinoma (HCC) cell line	Colon cell line	1496
842	Colon carcinoma (HCC) cell line II	Colon cell line	1744
841	HCC cell line (metastasis to liver in mouse) II	Adenocarcinoma cell line	1696
1447	NCI_CGAP_Co14	Adenocarcinoma	7122
3091	CT0214	Adenocarcinoma	1152
5351	NIH_MGC_15	Colon cell line	
2721	NCI_CGAP_Sub4	Pooled colon tumours	4188
486	Stratagene colon (#937204)	T84 colon cell line	7864
1079	NCI_CGAP_Co8	Colon tumour	9422
988	NCI_CGAP_Co12	Pooled colon tumours	2478
987	NCI_CGAP_Co9	RER+ colon tumour	3272
956	NCI_CGAP_Co10	RER+ colon tumour	3666

Table 2
Samples used for validation of in silico methods

Histology	Location	Size (mm)	Age	Sex
Normal	Sigmoid		66	Female
Normal	Descending		72	Male
Normal	Caecum		52	Female
Tubular adenoma	Sigmoid	<10	66	Female
Tubular adenoma	Caecum	<10	52	Female
Tubular adenoma	Sigmoid	<10	64	Male
Tubulo-villous adenoma with moderate dysplasia	Descending	<10	49	Female
Tubulo-villous adenoma with high-grade dysplasia	Sigmoid	12	63	Female
Tubulo-villous adenoma with moderate dysplasia	Descending	<10	72	Male
Adenocarcinoma	Rectum		79	Male
Adenocarcinoma	Sigmoid		58	Female
Adenocarcinoma	Transverse		62	Male

designed and synthesised for silencing both ETV4 and Myeov (Qiagen Inc., CA, USA). The siRNA had the following sequences: ETV4 sense, 5′-UGA AAG CCG GAU ACU UGG AUU-3′; ETV4 antisense 5′-UCC AAG UAU CCG GCU UUC AUU-3′; Myeov sense, 5′-GGA UGU AAG UUA UCA ACU A-3′; Myeov antisense, 5′-UAG UUG AUA ACU UAC AUC C-3′. A chemically synthesised non-silencing siRNA duplex with the following sequence; sense, 5′-UUC UCC GAA CGU GUC ACG U-3′; antisense, 5′-ACG UGA CAC GUU CGG AGA A-3′ that had no known homology with any mammalian gene was used to control for non-specific silencing events. Gene knockdown was achieved in T84 cells. Briefly, 4×10^4 cells were incubated under standard conditions overnight. Five micrograms of each siRNA was then mixed with 30 μl of RNAifect (Qiagen) and was added dropwise. Cells were incubated for 48 h again under standard conditions before being assayed.

Cell proliferation. To assess the effect of separate ETV4 and Myeov knockdown on cellular proliferation, 4×10^4 cells were added with 20 μl MTS reagent (Promega) to each well of a 96-well plate in triplicate. Absorption (at 490 nm) of each well was then read at 150 min using a microplate reader.

In vitro invasion. Biocoat invasion chambers (BD Sciences, USA) were used to investigate the effect of ETV4 and Myeov knockdown separately on the invasive capacity of T84 colon cancer cells. Briefly, 4×10^5 cells were seeded into the upper chamber and allowed to invade for 48 h under standard conditions. Following incubation, cells that had invaded through the basement membrane were fixed and stained before the membrane was removed and mounted on a slide for microscopic assessment. Invasive cells were visualised at 40× magnifications in five random fields and the number of cells in each field counted.

Results

Computational data-mining identified 163 known genes with enhanced expression in colon cancer libraries

The 15 colon cancer cDNA libraries were compared with 110 normal tissue libraries, including normal colon library. Digital Differential Display identified 205 known genes as being preferentially expressed in colon cancer (see Supplementary data). This included (a) 42 genes whose altered expression in colonic neoplasia had previously been described, and (b) 162 genes whose expression in this setting had not previously been described (Supplementary data). Functional classification of the identified genes revealed a significant number of genes associated with oncogenesis (i.e., ARHGEF5 and GRB7), signal transduction (i.e., CEACAM6 and LRP5), and cell proliferation (i.e., CDK4 and GRN); key activity indices in neoplasia. We selected the 15 genes displaying the highest relative expression in colon cancer for further study; nine of these had previously been disease associated (Table 3).

ETV4 and MYEOV expression is quantitatively increased in colonic neoplasia

We selected two genes, MYEOV and ETV4, for further study on the basis of their significantly enhanced expression in colon cancer cDNA libraries ($p < 0.05$). Myeov and ETV4 had 20- and 14-fold increased representation in colon cancer cDNA libraries when compared to normal libraries. There were no previous reports of their role in colonic neoplasia. ETV4 mRNA levels were significantly increased in colonic neoplasia in comparison to normal colonic tissue ($p < 0.05$) (Fig. 1a). MYEOV expression was elevated in neoplastic colon in comparison with normal tissue (Fig. 1b). Expression levels were greatest in carcinomas, suggesting a role in the latter stages of the polyp-cancer sequence.

Inhibition of ETV4 and MYEOV impairs colon cancer cell function

T84 colon cell lines were transfected with siRNA for ETV4 and MYEOV. Transfection of ETV4 siRNA resulted in 71% decreased ETV4 mRNA levels (Fig. 2a). This knockdown resulted in 90% decreased colon cancer cell proliferation in comparison with control cells ($p < 0.05$) (Fig. 3a). In vitro cell invasion was decreased by 67% using ETV4 siRNA (Fig. 3b). Transfection of MYEOV siRNA into T84 cells resulted in a 74% decrease in Myeov expression in comparison with control cells ($p < 0.05$) (Fig. 2b).

Table 3
Genes differentially expressed in colon cancer libraries (top 15 out of 162—see Supplementary material for complete list)

Accession No.	Cluster	Name	Expression	Ontology
NM_001644.2	560	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1)	Exclusive	mRNA editing
NM_001804.1	1545	Caudal type homeo box transcription factor 1 (CDX1)	Exclusive	Histogenesis
NM_005814	143131	Glycoprotein A33 (transmembrane) (GPA33)	Exclusive	Cell adhesion
NM_001986.1	77711	Ets variant gene 4 (E1A enhancer binding protein, E1AF) (ETV4)	20	Oncogenesis
NM_001265.2	77399	Caudal type homeo box transcription factor 2 (CDX2)	20	Oncogenesis
NM_138768.1	116051	Myeloma overexpressed gene positive multiple myelomas (MYEOV)	14	
NM_004963.1	1085	Guanylate cyclase 2C (heat stable enterotoxin receptor) (GUCY2C)	13	
NM_024017.3	86327	Homeo box B9 (HOXB9)	6	
XM_032721.3	109358	ATPase, Class V, type 10B (ATP10B)	5	
NM_033266.1	114905	ER to nucleus signalling 2 (ERN2)	4	
NM_019010.1	84905	Cytokeratin 20 (KRT20)	4	Digestion
NM_005310.1	86859	Growth factor receptor-bound protein 7 (GRB7)	4	Oncogenesis/signalling
NM_001738.1	23118	Carbonic anhydrase I (CA1)	4	
NM_004306.1	181107	Annexin A13 (ANXA13)	3	
NM_007028.2	91096	Tripartite motif-containing 31 (TRIM31)	3	

Exclusive, no transcripts in normal libraries; expression, fold increase in absolute number of transcripts in colon cancer libraries.

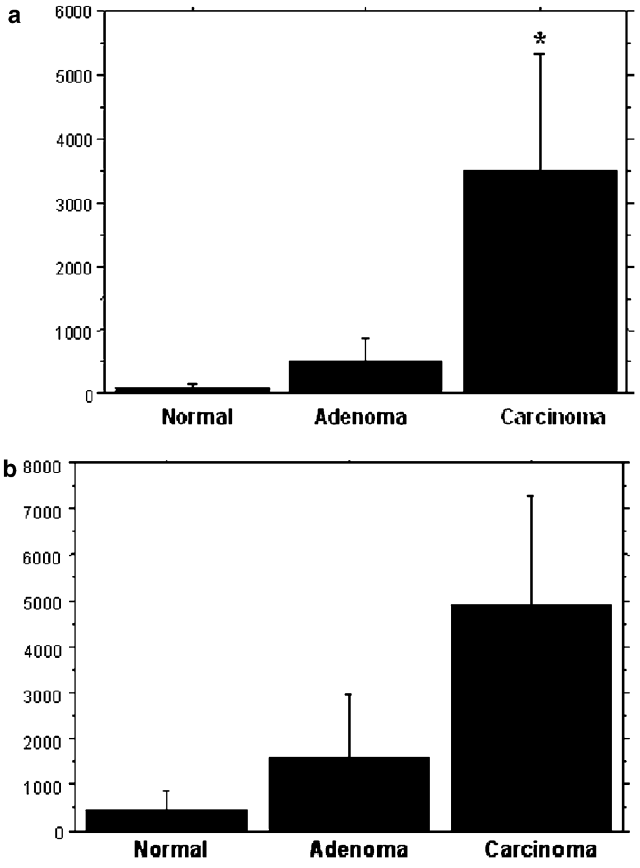

Fig. 1. Increased expression of ETV4 and MYEOV in colonic neoplasia detected using quantitative RT-PCR. Quantitative real-time PCR was employed to determine the expression of ETV4 (a) and MYEOV (b) in samples of normal colon ($n = 3$), adenoma ($n = 6$), and carcinoma ($n = 3$). ETV4 expression was significantly increased in carcinoma when compared to normal tissue. Increased MYEOV expression was detected in cancer in comparison with normal tissue. The graphs show expression in arbitrary units (y axis) of each gene relative to GAPDH in the tissue samples (x axis). $^*p < 0.05$.

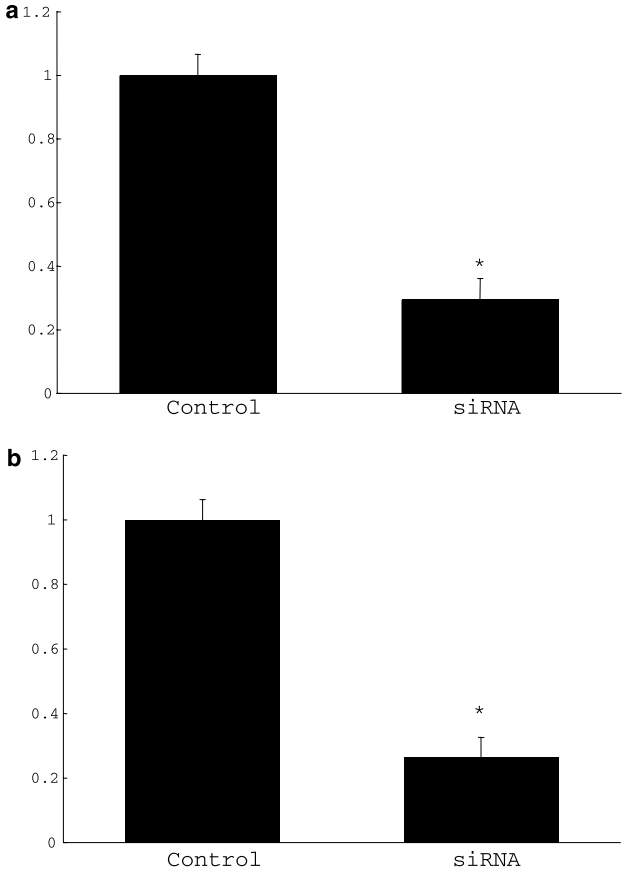

Fig. 2. Confirmation of siRNA-mediated gene knockdown using quantitative real-time PCR. Real-time PCR was used to confirm decreased (a) ETV4 and (b) Myeov mRNA levels in cells treated with siRNA in comparison with control cells. $^*p < 0.05$.

This knockdown resulted in a significant 48% decrease in cell proliferation ($p < 0.001$) (Fig. 4a). MYEOV knockdown reduced in vitro colorectal cancer cell invasion by 36% (Fig. 4b).

Discussion

This study demonstrates the application of an integrated computational biology approach to the identification of novel genes associated with colon cancer and their functional roles. Unlike other approaches such as oligonucleotide microarrays and SAGE, the bioinformatic screening tool used is freely available and dependent only on the available cDNA libraries.

The description of the functional effects of inhibition of ETV4 and MYEOV on colon cancer cell lines suggests a mechanism for their role in colonic carcinogenesis. ETV4 has recently been reported to be associated with overexpression of matrix metalloproteinases in colorectal cancer [7]. Our functional demonstration of its association with invasion and cell proliferation further implicates this transcription factor in colonic carcinogenesis. Of note, a novel germ-line mutation within a sequence homologous to the consensus sequence for ETV4 was identified in patients with hereditary non-polyposis colon cancer [8]. It is likely that activation of ETV4 transcription factor by gain-of-function mutation promotes invasion by stimulating transcription of metalloproteinases, iNOS and COX-2 [9].

The implication of Myeov in cell proliferation in colon cancer is novel. Myeov is a transforming gene at 11q13 that was originally described in myeloma, and is frequently amplified in breast and oesophageal carcinoma cell lines [10–12]. It has been described in this setting in conjunction with cyclin D1 amplification. This study has demonstrated expression of Myeov in advanced neoplasia, suggesting that the increased expression of this putative oncogene is associated with cell proliferation. MYEOV sequences were not present in UniGene libraries of colon cancers that are replication error positive (RER+), i.e., harbour alterations in the mismatch repair pathway. We thus hypothesise that transcription of MYEOV may occur via an alternative pathway to the mismatch repair pathway that characterises 25% of colon cancers.

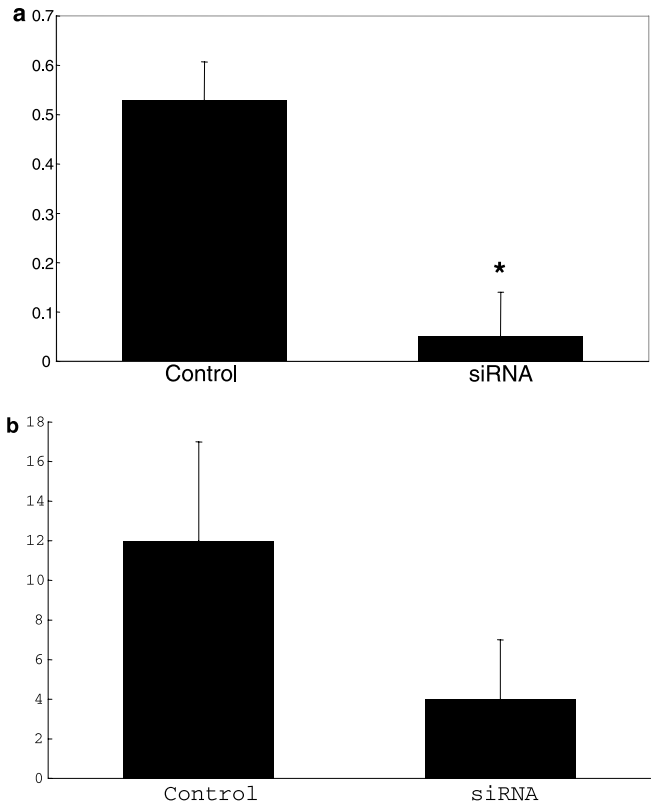

Fig. 3. Functional effects of ETV4 inhibition by siRNA. Inhibition of ETV4 translation by siRNA transfected into T84 colon cancer cells impaired cell proliferation in these cells compared to control cells (a). Inhibition of ETV4 translation also impaired cell invasion in T84 cells as measured by invasion of cells across the membrane of an invasion chamber (b). $*p < 0.05$.

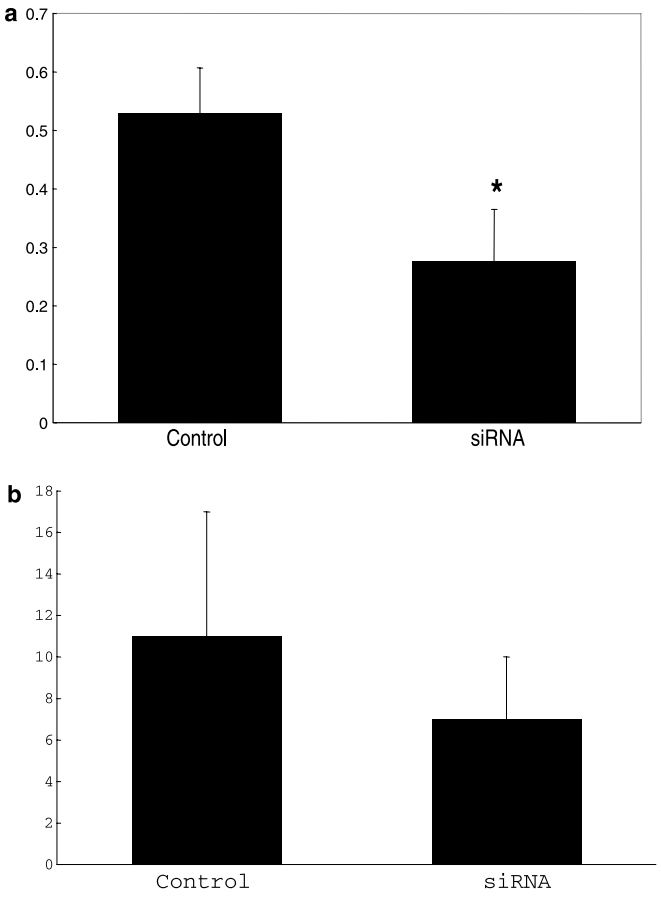

Fig. 4. Functional effects of MYEOV inhibition by siRNA. Inhibition of MYEOV translation by siRNA transfection of T84 colon cancer cells impaired cell proliferation in comparison to control cells (a). Inhibition of MYEOV translation inhibited the invasion of T84 cells (b). $*p < 0.05$.

The significance of results generated by DDD is dependent on the quality of the input data. Information on the source of tissue is sometimes incomplete, as are descriptions of whether the tissue is bulk or micro-dissected. The relatively small normal colon library meant that the majority of the genes are derived from a comparison with normal adult tissue. Finally, as the databases are based on EST sequencing, there is a bias towards highly expressed genes. However, the conservative statistical test used by DDD, and the large size of the comparison libraries used, reduces the potential for false positive results. Proof-of-principle for the technique lies in the large number of identified genes involved in oncogenesis, many of which are well-characterised markers of colonic nesoplasia, i.e., cytokeratin 20. Although the majority of the other matches were with hypothetical proteins, these may yet prove to be factors in carcinogenesis.

In aggregate, this study validates the use of high-throughput data-mining to identify genes altered in colonic neoplasia. It has demonstrated a novel association between MYEOV cell proliferation and invasion in colon cancer cell lines, and independently confirmed the role of ETV4 in invasion in colon cancer. This template could be used to examine other tissue libraries and disease states and may provide an exciting gene discovery route in studies of human cancers.

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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.bbrc.2006.04.094.

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