The Transcript Expression and Protein Distribution Pattern in Human Colorectal Carcinoma Reveal a Pivotal Role of *COM-1/p8* as a Tumour Suppressor

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Abstract. *Background and Aims: COM-1(P8) is thought to play a role in the formation of metastases. This appears from current evidence to be different in various types of solid tumours. We aimed to examine the role COM-1 played in the development of colorectal cancer. Materials and Methods: The expression of COM-1 mRNA was examined using a quantitative polymerase chain reaction (PCR) technique together with immunohistochemistry to examine expression and distribution of the COM-1 protein in human colorectal carcinoma and matched normal colorectal mucosa. Results: COM-1 was expressed in 22.8% of normal colorectal mucosa samples and the expression in these tissues was 54.9 copies of COM-1 transcript per sample. In tumour tissues, 43.6% of samples expressed COM-1, at a level of 98.9 copies of COM-1 transcript per sample (p=0.012). Normal tissues demonstrated strong nuclear and peri-nuclear staining for COM-1 on immunohistochemistry (IHC) and in tumour tissues, the level of staining was found to be much greater, with a greater degree of cytoplasmic staining and little nuclear staining. Early-stage tumours showed a greater degree of staining on IHC compared to those at an advanced stage of disease. Conclusion: COM-1, although overexpressed at the messenger level, appears to be distributed in a cytoplasmic fashion at the protein level in tumours. Tumours at advanced stage express COM-1 protein to a lesser extent than their early-stage counterparts.*

COM-1, candidate of metastasis-1, otherwise known as p8, is a primarily nuclear protein initially discovered within the last decade by the differential display of cDNA from breast cancer metastases within the brains of athymic nude rats. Although it was initially thought to play a role in the development and maintenance of metastatic tumours and to have some mitogenic activity (1-3), subsequent investigations have demonstrated a much more diverse role in cancer and cell growth for COM-1.

The expression of COM-1 in various malignant tumours with a variable pattern of expression has been identified. In breast cancer, for example, *COM-1* mRNA was shown to be expressed in cancer tissues more frequently than in the normal tissues of the same patient (1). In pancreatic cancer, some studies have associated overexpression of COM-1 with pancreatic cancer tissues (4, 5) and others have found the reverse, in that knockout of *COM-1* in pancreatic cancer cell lines resulted in increased growth rates (6). Fibroblasts transfected with *COM-1* have been shown to grow more slowly and those with *COM-1* knockout grow more rapidly and are more resistant to chemically induced apoptosis (7, 8). In thyroid cancers, *COM-1* has been found to be overexpressed in papillary and follicular tumours but to a much lower degree in anaplastic tumours, this is together with a change in location of COM-1 expression on immunohistochemical (IHC) staining from nuclear (found in normal tissue and follicular tumours) to cytoplasmic (in papillary and anaplastic tumours) locations (9). In prostate tissues, we have shown that cancer tissues have significantly reduced expression of COM-1 protein compared to normal prostate tissue whereas prostate cancer cell lines widely expressed COM-1. Knockout of *COM-1* in these cell lines resulted in accelerated growth of each line and forced overexpression resulted in retardation of both growth rate and invasiveness (10).

In addition to this there is evidence that some tumour inhibitory agents (1,25-dihydroxycholecalciferol and transforming growth factor (TGF)-β) may up-regulate the expression of COM-1 and result in cell growth arrest (11, 12). These findings may suggest that COM-1 probably

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behaves very differently from the role suggested by earlier studies which may reflect the complexity of the molecules interactions and role.

We have observed that no studies have examined the expression of *COM-1* in tumours of the lower gastrointestinal tract and as such have set out to examine quantitatively the expression of *COM-1* in carcinomas and matched normal tissues of the colon and rectum and to examine the localisation of COM-1 protein within the tissues.

Materials and Methods

Materials. RNA-extraction kits and reverse transcription (RT) kits were obtained from AbGene (Guildford, Surrey, UK) and Sigma-Aldrich (Dorset, England, UK). Polymerase chain reaction (PCR) primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesised by Invitrogen (Paisley, Scotland, UK). Molecular biology grade agarose and DNA ladder were sourced from Invitrogen, whilst the master mix for routine PCR and customised master mix for quantitative PCR came from AbGene. Universal Z-probe for qPCR was supplied by Intergen (Oxford, United Kingdom). Rabbit anti-human COM-1 (p8) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase conjugated anti-rabbit antibodies were acquired from Sigma-Aldrich UK (Gillingham, Dorset, UK) and a biotin universal staining kit was from Vector Laboratories (Nottingham, UK).

Cells and tissue samples. Fresh colon carcinoma tissues (n=94) and a comparative normal background tissue sample from the same patient (n=80) were collected immediately after surgery and stored in a deep freezer until use. Normal tissue samples were collected as distant as possible from the neoplasm normally from the proximal resection margin. Details of histology and clinical outcome were obtained from pathology reports and patient records (Table I). The presence of carcinoma tissue in carcinoma samples and the absence of carcinoma from normal comparison samples was confirmed by a consultant pathologist using standard H&E staining techniques. Regional Ethics Committee approval was obtained for collection of tissues and tissues were only collected from patients after obtaining fully informed consent (Study Number: 05/WSE03/92).

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut to thicknesses of 6 µm and kept for IHC and routine histology. A further 15-20 sequential sections were mixed and homogenized using a hand-held homogeniser in ice-cold RNA extraction solution and total cellular RNA extracted using a standard technique. The concentration of the RNA was determined using a UV spectrophotometer. Reverse transcription was conducted using an RT kit with an anchored oligo-dT primer (supplied by Sigma-Aldrich). Reverse transcription was carried out using 1 µg total RNA in a 96-well plate. The quality of the RT reaction and consistency of sample loading were verified by amplifying a house keeping gene, β-actin.

Quantitative analysis of COM-1. The level of the *COM-1* transcripts from the prepared cDNA was determined by real time quantitative PCR, based on the Amplifluor™ technology as recently reported (13,14), modified from a method reported previously (15). Briefly, a pair of PCR primers was designed using Breacon Designer software

Table I. *Clinical and pathological information of the patients.*

Clinical information	Number (n)	Percentage (%)
Dukes' stage		
A	15	19.5
B	32	41.6
C1	29	37.7
C2	1	1.3
T stage		
T1	8	10.4
T2	10	13
T3	40	51.9
T4	19	24.7
Differentiation		
Well-differentiated	10	13.0
Moderately differentiated	53	68.8
Poorly differentiated	14	18.2
Nodal status		
Negative	45	58.4
Positive	32	41.6
Gender		
Male	47	52.2
Female	43	47.8

(version 2) (sequence given in Table II). To one of the primers (an antisense primer is routinely used in our laboratory) an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3') which is complementary to the universal Z probe[15] (Intergen) was added. A Taqman detection kit for β-actin was purchased from Perkin-Elmer (Waltham, Massachusetts, USA). The reaction was performed using: Hotstart Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol of reverse primer which has the Z sequence, 10 pmol of 6-carboxyfluorescein (FAM)–tagged probe (Intergen), and cDNA from approximately 50 ng RNA (calculated from the starting RNA in the reverse transcriptase reaction). The reaction was carried out using IcyclerIQ™ (BioRad, Hemel Hempstead, England, UK) which is equipped with an optical unit that allows real time detection of the 96 reactions under the following conditions: a single cycle of 94˚C for 12 mins, 50 cycles of: 94˚C for 15 seconds, 55˚C for 40 seconds and 72˚C for 20 seconds and a final cycle of 72˚C for 10 mins. The levels of the transcripts were generated using an internal standard that was simultaneously amplified with the samples, and are shown here as levels of transcripts based on equal amounts of RNA. Levels of transcripts expressed in normal matched colorectal mucosal tissue samples and tumour samples can then be compared in two ways: firstly by comparison of absolute levels of transcripts in each sample and tissue (carcinoma *vs*. normal), secondly by comparing those carcinoma samples that express *vs*. non-expressors or overexpression *vs*. underexpression in each tumour sample when compared with its matching normal mucosal sample.

IHC staining of the COM-1 protein. The staining was based on a method we described previously (16). The frozen sections of colorectal tumour and normal tissues from the distal end of resection (proven negative for tumour) were cut to a thickness of 6 µm using a cryostat. The sections were mounted onto Super-Frost Plus microscope slides, air dried and then fixed in a mixture of 50%

Table II. *Primers used in the current study.*

	Sense primers (5'-'3)	Antisense primers (5'-'3)
COM-1	CCTGGATGAATCTGACCTC	ACTGAACCTGACCGTACACAAGCAGCTTCTCTCTTGGTG
β-Actin	ATGATATCGCCGCGCTCG	CGCTCGTGTAGGATCTTCA

Z sequence for quantitation is underlined.

acetone and 50% methanol. The staining procedure was completed simultaneously for all stained sections to ensure comparison. The sections were then placed into Optimax wash buffer for 5-10 mins to rehydrate. After a further 40 min incubation in a 1% horse serum blocking solution, the sections were probed with the primary antibody (diluted 1:100 for anti-COM-1). After extensive washings, the sections were incubated for 30 min with the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin; Dako, Ely, Cambridgeshire, UK). Following further washings, avidin-biotin complex (Vector Laboratories, CA, USA) was then applied for 5 min following which diamionobenzidine chromogen (Vector Laboratories, Peterborough, England, UK) was then added to the sections which where then incubated in the dark for 5 min. Sections were counterstained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing with xylene and mounting under a coverslip.

Statistical analysis. The statistical analysis was carried out using the Mann-Whitney *U*-test, the Kruskal-Wallis test and the Chi-square test using Minitab 10.2 and SPSS (Statistical Package for Social Sciences) 14.0 software (Minitab Inc, PA, USA). Statistical difference was considered to be significant at $p<0.05$.

Results

COM-1 transcripts in tumour and normal mucosal tissues. *COM-1* was expressed in only 18 (22.8%) out of 80 normal colorectal tissue samples the median level of expression in these tissues was 54.9 copies of *COM-1* transcript per sample. In the tumour tissues, 41 (43.6%) out of 94 tumour samples expressed *COM-1* in the analysis, of these, the median level of *COM-1* expression was 98.9 copies. Comparing the levels of expression in those tissues samples that expressed *COM-1, COM-1* in tumour tissues was significantly overexpressed (Mann-Whitney U-3058, $p=0.012$) compared to the normal tissues. The number of samples that expressed *COM-1* in the tumour tissue group was found to be significantly higher than that in the normal tissue group (Chi-square 8.289, $p=0.004$).

With regard to the T stage, there was a significant decrease in the percentage of tumours overexpressing *COM-1* compared to their matched normal counterpart with increasing T stage of the tumour from 100% of T1 and T2 tumours overexpressing compared to normal, 90% of T3 tumours overexpressing and falling further to only 50% of T4 tumours overexpressing *COM-1* compared to their matching normal tissues (Chi-square 8.548, $p=0.036$). However, although absolute median levels of expression in T1 were 3770 compared to 247 in T4 disease, this was not shown to be significantly different on Mann-Whitney analysis.

Similarly, with N stage there was a decrease in tumours overexpressing COM-1 compared to normal from 88.9% of tumours in N0 disease to 55.6% in N2 disease, although this was not significant on Chi square test ($p=0.091$). Again with M stage, overexpression dropped from 89.7% in M0 disease to only 60% in M1 disease, although this was not statistically signficant ($p=0.211$). With histological grade, levels of overexpression also decreased with worsening degree of tumour differentiation from 100% overexpression in well-differentiated tumours to 83.3% in moderately differentiated tumours and only 51.7% in poorly differentiated tumours. However, again this difference failed to reach a significant difference ($p=0.151$).

IHC of COM-1 expression in tumour and normal mucosa. Normal tissues on IHC staining for COM-1 protein demonstrated strong nuclear and peri-nuclear staining of the protein, with little or no cytoplasmic staining seen, stromal staining was absent and there was no evidence of vascular or lymphovascular staining (Figure 1). In tumour tissues however, the overall level of staining was found to be much greater, with a much higher degree of cytoplasmic staining and little nuclear or peri-nuclear staining (Figure 1). Again no stromal, vascular or lymphovascular staining was identified.

Interestingly, on further analysis, early-stage tumours showed a greater degree of staining on IHC when compared to their matched normal tissues (Figure 2), whereas those tumours which were at an advanced stage of disease showed a much lower level of staining for COM-1 overall when compared to their matched normal tissues (Figure 3).

Discussion

To our knowledge, this study is the first to examine the quantitative expression of *COM-1/P8* mRNA transcripts in both normal colorectal mucosa and colorectal carcinoma tissue samples, in addition to examining the staining pattern and levels of COM-1/p8 protein expression in the same tissue samples.

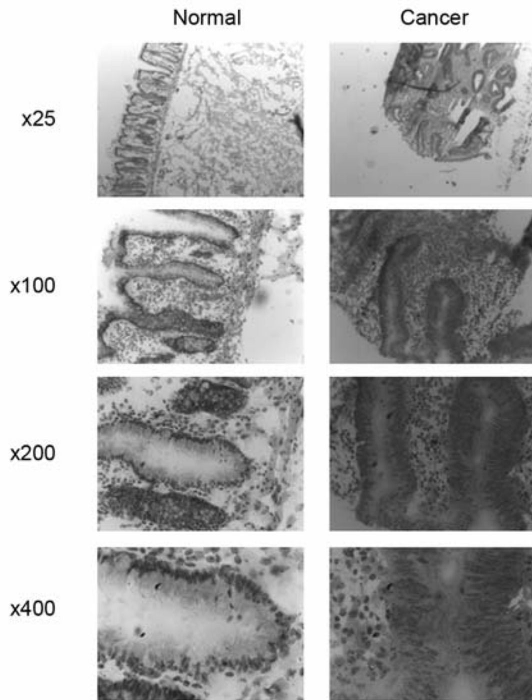

Figure 1. *Immunohistochemical staining of COM-1 protein in a panel of normal and tumour tissues.*

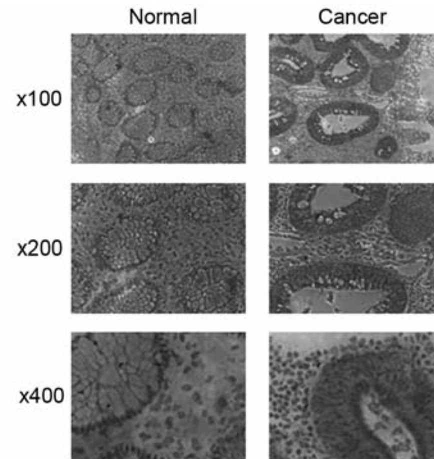

Figure 2. *Immunohistochemical staining of COM-1 protein in early stage colon cancer. Matched normal and tumour tissues from the same patient are shown.*

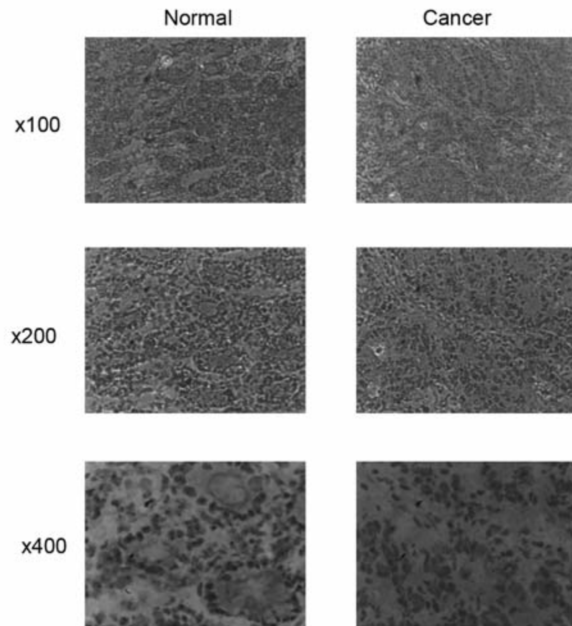

Figure 3. *Immunohistochemical staining of COM-1 protein in advanced stage colon cancer. Matched normal and tumour tissues from the same patient are shown.*

COM-1 has been demonstrated to be expressed in normal colorectal mucosa at either low levels or not to be expressed at all; however within colorectal carcinoma tissue COM-1 is found to be expressed more frequently and when expressed its level of expression is higher than that found in normal colorectal mucosa. This pattern of overexpression of COM-1 has been demonstrated as discussed above in carcinoma tissues of papillary and follicular thyroid cancer (9), breast cancer (19) and pancreatic cancer (4), but the reverse has also been demonstrated in prostate cancer and breast cancer tissues (1). The expression of COM-1 in cancer cell lines appears to be even more irregular in that in some cancer tissues, such as in pancreatic cancer, COM-1 is overexpressed but commonly used cell lines for pancreatic cancer express COM-1 at a much lower level than normal tissues. In contrast, prostate cancer tissues underexpress COM-1 compared to normal tissue but prostate cancer cell lines widely overexpress COM-1 (10). In addition to this, it would appear from the evidence available that forced overexpression in genetically manipulated cell lines (pancreatic and prostate cancer cell lines and fibroblast cell lines) results in reduced growth rates and reduced invasiveness, and knock-out of *COM-1* in the same cell lines results in their increased growth and invasiveness (4, 7, 10).

In this study, another interesting feature of COM-1 expression has been demonstrated. An association was found between lower than expected levels of COM-1 expression and higher stage of primary tumour such that the levels of COM-1 expression decreased with increasing T stage of the tumours to a degree of statistical significance. In addition, there was a non-significant trend toward lower levels of COM-1 expression in higher N stage disease, higher M stage disease, and worsening degree of tumour differentiation consistent with the findings in the T stage analysis.

With regard to the COM-1 protein level and pattern of COM-1 distribution throughout the tissues, COM-1 was

expressed at a much lower level and was confined to the nucleus and peri-nuclear cytoplasm in normal tissues but in carcinoma tissues was found to be located in the cytoplasmic areas, having a much lower nuclear expression. This is consistent with the pattern shown by papillary and anaplastic thyroid carcinomas (9). Although the exact role that COM-1 plays in the regulation of cell growth and mobility in normal tissues is poorly understood from most of the evidence, it appears that the cellular location of the protein is critical to its role in the control of cellular functions. For example, COM-1 has been demonstrated to interact with nucleus tumour suppressor proteins peroxisome proliferator activated receptor gamma co-activator-1 (PGC-1), oestrogen receptor beta (ER-β) and p300 (10, 17, 18). PGC-1 and ER-β are virtually exclusive nuclear proteins and regulate the growth of cells. Thus, it would not be unreasonable to assume that in normal colon tissues (similar to prostate, breast and thyroid), COM-1 is confined to the nucleus and acts as potential tumour suppressor.

In this situation, it would be consistent to believe that following the development of a carcinoma, the COM-1 molecule is initially overexpressed in a protective manner, limiting the disease to lower stage or aggressiveness. However, as the tumour becomes increasingly de-differentiated, there may be the loss of (or progressive reduction in function of) COM-1 protein resulting in increased aggressiveness of tumour; this would lead to the development of a more advanced local disease, increasing the likelihood of nodal and distant metastases. Furthermore, mechanism(s) for the translocation of COM-1 to the nucleus may also be impaired, preventing the occurrence of nuclear COM-1. The cytoplasmic COM-1 protein may either fail to interact with nuclear tumour suppressing proteins or may itself trigger cellular events that may facilitate the growth of cancer cells. In this case, COM-1 would be acting as a putative tumour suppressor gene in colorectal carcinoma, however, it cannot be denied that colorectal carcinoma tissues do overexpress COM-1 protein compared to their normal counterparts and, as such, the reverse may also be true. Further work would be needed to analyse the level of COM-1 expression in colorectal carcinoma cell lines and to assess the effect of forced COM-1 overexpression and *COM-1* knock-out on the growth rate and invasiveness of such cell lines before a better understanding of the role of COM-1 in colorectal carcinoma is gained. This is currently ongoing in our laboratories.

In conclusion, there is a pattern of aberrant expression of *COM-1* in human colon cancer at both the protein and message levels. While the *COM-1* transcript is increased in tumour tissues compared with normal tissues, low levels of *COM-1* transcript are seen in aggressive tumours. Finally, the intriguing cellular distribution pattern of COM-1 protein in colon tissue reveals its cellular location may have an impact on its regulatory role in colonic epithelial cells.

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