REG GENE EXPRESSION IN CANCER TISSUE

The invention relates to a method and/or marker for determining the aggressive nature of cancer tissue for use particularly, but not exclusively, for diagnostic, prognostic or drug screening purposes; and a kit for use therefor.

Given our current knowledge, the invention is thought to have particular application in determining a predisposition towards and/or the aggressive nature of gastrointestinal, pancreatic, colorectal, prostate cancers. However it is not intended that the invention should be limited thereto, rather, we consider that the invention has wide application in a determination of the aggressive nature of any type of cancer tissue.

In this application by the term aggressive nature we mean the probability of an individual experiencing disease relapse following curative treatment and/or surgery.

A number of techniques have been developed in order to try and predict the aggressive nature of cancer tissue and insofar as the gastrointestine, and more precisely colorectal cancer, is concerned current practice involves subjecting a resected specimen to routine pathological staging using what is known as the Dukes' classification. This classification involves an examination of the specimen in order to determine the penetrative nature of a tumour. Essentially, there are three classifications A, B and C. A characterises tumours that have been confirmed to within the bowel wall, B characterises tumours that have penetrated the bowel wall, and C characterises tumours that have spread to local lymph nodes or distant sites. There are very few

instances where complete cure is feasible when metastases are present but in patients with lymph node metastases (Dukes' C disease) studies have shown an advantage in administration of adjuvant chemotherapy using 5-fluorouracil and a biological modifier of 5-fluorouracil activity. It is, however, unclear which of those patients with earlier stage diseases (Dukes' stages A and B) would benefit from adjuvant chemotherapy. Whilst the majority of patients with early stage disease are likely to survive free of disease recurrence for five years, a small but significant proportion of these suffer disease relapse and die of the effect of their cancers. It would therefore be highly desirable to develop a method of predicting those patients with early stage disease who are likely to relapse in order to offer adjuvant chemotherapy to this selected group. In addition, if such a marker of aggressive tumour behaviour was found, it is feasible that patients with tumours not exhibiting this feature may be found to follow a more benign clinical course following resection and may therefore be spared the unpleasant side-effects of chemotherapy. Furthermore, targeting of adjuvant chemotherapy in this manner would have significant resource implications and could result in substantial cost savings.

The identification of a suitable marker for determining not only the aggressive nature of gastrointestinal cancers, but also other cancers would be a significant development in the treatment of cancer patients.

Moreover, such a marker would also be of significant benefit in determining the efficacy of cancer drugs. This is because individuals identified as either positive or negative for the marker could be suitably selected for clinical trials. Thus, the data from such trials would not be compromised by the use of a distorted sampling population, in other words poor results could be assessed having regard to the predicted aggressive nature of the disease to be

treated.

We believe we have identified a marker for determining the aggressive nature of cancer tissue and this marker is the pancreas regenerating gene (*REG*) product.

The pancreas regenerating gene (*REG*), identified by Terazono *et al*[1] in 1988, is expressed in regenerating pancreatic islet cells in the rat but not in normal islet cells. The human homologue to rat *REG* cDNA in pancreas encodes a 166 amino acid protein with a signal sequence. This sequence derived from *REG* cDNA contains a previously described 144 amino acid sequence of pancreatic stone protein (PSP)[2] and the partially-determined 45 amino acid sequence of pancreatic stone protein.[3] The human *REG* gene has been mapped to chromosome 2p12.[4, 5] It is a pleiotropic gene with a role in modulating cell replication growth and maturation. The protein product, lithostathine, is secreted by the normal exocrine pancreas and inhibits the growth of calcium carbonate crystals. Levels of this protein in pancreatic juice have been found to be reduced in chronic pancreatitis.[6]

Previous studies using Northern blot analysis[7] detected *REG* mRNA in normal pancreas, gastric mucosa and kidney, but not in the liver, spleen, brain, thyroid gland, submandibular gland, oesophageal mucosa, rectal mucosa or lymphocytes.[7] *REG* mRNA was detected in various levels in two gastric carcinomas, 5 out of 6 colonic carcinomas, a colonic polyp and a rectal carcinoma, despite the absence of *REG* mRNA in any non-tumour mucosa of the colon and rectum. However, despite this detection no one has previously considered that *REG* gene product and in particular *REG* mRNA may be suitable as a marker for determining the aggressive nature of a

cancer. This is because nobody has followed up these studies with a view to linking disease relapse with *REG* gene expression.

However, we have undertaken this work and we have found a coloration between *REG* gene expression and a wide range of cancers. This information is of particular importance where one is concerned, an extremely aggressive cancer, with is concerned, such as pancreatic cancer. Indeed, the outlook for patients presenting with pancreatic cancer is exceptionally poor , and fewer than 1% of patients will be alive five years following diagnosis. Surgery offers the only hope of cure, and for patients with localised disease, surgical resection may be possible. Studies examining the systemic dissemination of small numbers of tumour cells from other gastrointestinal tumours suggest this feature to be associated with a poor prognosis. Whilst the prognosis in pancreatic cancer is frequently poor following potentially curative surgery, the presence or absence of disseminated micrometastases may well be of significance in the planning and evaluation of adjuvant therapy and the establishment of accurate prognosis. Previous immunohistochemical studies in pancreatic cancer have shown a high rate of bone marrow micrometastases present at the time of initial surgery, and related this finding to a poorer disease-specific outcome (8). We have developed a RT-PCR based approach to the detection of bone marrow micrometastases from pancreatic tumours, The PCR reaction amplifies cDNA for the gene REG which is constitutively expressed in pancreatic acinar tissue and encodes for the secreted protein lithostathine. Moreover, we have shown that *REG* gene products are present in peritoneal washing from patients with pancreatic cancer.

We have also developed a RT-PCR based approach to the detection of bone marrow micrometastases in patients with colorectal cancer.

In addition, we have shown that detection of *REG* mRNA in normal-appearing mucosa around colorectal cancers may be used to determine prognosis and thus corresponding treatment.

In addition, we were shown a correlation between *REG* gene expression products and malignant tissues in prostate tissue and also a correlation between the expression of this gene and individuals suffering from or known to suffer from ulcerative colitis a disease known to predispose one to cancer.

Given the above, it can be seen that products of the *REG* gene serve as markers for not only establishing the tendency towards, but also the aggressive nature of cancer tissue.

It is therefore an object of the invention to provide a method for determining a tendency towards or the aggressive nature of cancer.

It is yet a further object of the invention to use *REG* gene transcription and/or translation as a marker for aggressive cancers, and more particularly, *REG* gene expression products as such markers.

It is yet a further object of the invention to provide a kit for determining the aggressive nature of cancer tissue.

According to a first aspect of the invention there is therefore provided a method for determining a tendency of a tissue to become cancerous or the aggressive nature of cancer tissue comprising examining said tissue and/or fluid associated with same to identify a product of *REG* gene transcription and/or translation.

In a preferred method of the invention said investigation involves the identification of *REG* mRNA alternatively, or in addition, it involves a determination of the *REG* gene protein.

In the instance where the protein is to be determined antibodies, monoclonal or polyclonal, may be used in order to bind to said *REG* protein. Advantageously, additional antibodies may be used to amplify the nature of the reaction and/or antibodies tagged with suitable labels may also be used.

In the instance where fluid is examined a blood sample may be taken and the amount of *REG* gene product determined and ideally compared with normal levels. Once this is done an elevated level of *REG* gene product in an individual suffering from, or known to have suffered from, cancer would be indicative of the existence of, or recurrence of, an aggressive cancer, respectively, and so appropriate action could be taken.

According to a further aspect of the invention there is provided the use of the *REG* gene, and more particularly, a product thereof for the determination of the aggressive nature of cancer tissue.

According to a yet further aspect of the invention there is provided a screening procedure for determining the efficacy of a selected therapeutic comprising;

a) examining cancer tissue and/or fluid associated with same from an individual who may take part in said screening in order to determine whether *REG* gene is transcribed and/or translated; and

b) depending upon the results of a) above including or excluding said individual from trials to investigate the efficacy of said therapeutic.

It will be apparent that in the instance where the efficacy of a therapeutic which is designed to treat particularly aggressive cancers is to be determined then individuals that are expressing the *REG* gene, or termed *REG* gene positive, will be selected. Conversely, individuals which do not express said gene will not be selected. Alternatively, where a therapeutic has been designed to treat less aggressive cancers, individuals who are *REG* negative will be selected for trials and individuals which are *REG* positive will not be selected. However in some instances a mixed population of individuals may be selected.

According to a yet further aspect of the invention there is provided a kit for determining the aggressive nature of cancer tissue comprising means suitable for identifying transcription and/or translation of the *REG* gene.

An embodiment of the invention will now be described by way of example only with reference to the following figures wherein;

Colorectal Cancers

Figure 1 shows the correlation between the currently used Dukes' classification and *REG* gene mRNA expression.

Figure 2 shows tumour recurrence data for individuals that are *REG* gene positive and *REG* gene negative.

Figure 3 is a graph showing the survival rate of individuals who are *REG* gene positive and *REG* gene negative.

Figure 4 shows two years survival data for individuals who are *REG* gene positive and *REG* gene negative.

Figure 5 shows cancer specific survival rate for individuals who are *REG* gene positive and *REG* gene negative.

Figure 6 shows survival in patients with REG-positive tumours of the colon or rectum with and without *REG* mRNA detected in bone marrow aspirates at the time of surgery.

Figure 7 shows a disease-free survival in patients with *REG*-positive tumours of the colon or rectum with and without *REG* mRNA detected in bone marrow aspirates at the time of potentially curative surgery.

Figures 8 and 9 shows the expression of the *REG* gene in both cancer tissue and tissue at a varying distance therefrom (i.e. 2, 4, 6 cm) and in normal-appearing mucosa (nm) situated at the margin.

Prostate Cancer

Figure 10 shows expression of *REG* mRNA in prostatic biopsy material (duplicated results).

Ulcerative Colitis

Figure 11 shows *REG* expression in mucosal biopsies in active inflammatory bowel disease, and in quiescent-appearing mucosa.

The use of *REG* gene products as suitable markers for determining the aggressive nature of cancer tissue was identified having regard to the following experiments.

COLORECTAL CANCERS

Materials and Methods

We obtained from our tissue bank 48 matched tumour and adjacent non-tumour mucosa samples collected at resection or surgery for colorectal cancer. Following snap freezing in liquid nitrogen at the time of collection these were stored at -80°C until analysis. Clinical follow-up was available on all 48 patients for a minimum period of two years.

RNA Extraction

Samples of mucosa were sliced from the frozen tissue blocks, ground to powder in an Eppendorf tube and immediately resuspended in 1ml of tetradecyltrimethylammoniumoxylate (Catrimox, Iowa Biotechnology Corps, Iowa, USA). Particulate remains were allowed to precipitate 5 minutes when the supernatant was aspirated for further analysis. The supernatant was centrifuged for 5 minutes at 1,000G forming an RNA pellet. The supernatant was discarded and the RNA pellet resuspended and mixed in 1ml of 2mol lithium chloride. Further centrifugation was carried out at 13,000G for 5 minutes. A total of 5 cycles of centrifugation and resuspension in lithium

chloride were carried out. The final resuspension was carried out in 1ml of 75% ethanol at 4°C. Following centrifugation at 13,000G for 5 minutes, the supernatant was discarded and the container inverted in a vacuum apparatus for 30 minutes. Any residual beads of liquid were aspirated from the container and the RNA pellet resuspended in 20μl of water, containing 1mmol DTT and 1 unit per μl RNAsin.

Reverse Transcription

10μl of the solution prepared as above was added to 10μl of reverse transcription mix containing 3mmol magnesium chloride, 1 x RT buffer (Promega, Madison, WI, USA), 1mmol each DNTP, 2.5μg per 100μl oligo DT, 1 unit per μl RNAsin, 600 units per 100μl MMLVRT. Reverse transcription was carried out at 42°C for one hour followed by a five minute period at 95°C.

PCR Reaction

PCR for *REG* cDNA was carried out only after satisfactory results were obtained from PCR reactions instigated to detect DNA contamination of RNA samples, using heat shock protein primers and cDNA formation using G3PDH primers.

Primers spanning introns were devised to amplify a 263bp product of *REG* cDNA (*REG* upper dAACATGAATTCGGGCAACC; *REG* lower dGGCACATCCTTCCATTTCT). *REG* primers and template (1μl RT reaction product) in a final volume of 10μl were overlaid with mineral oil and heated to 95°C for a hot start PCR. The reaction was initiated by adding

10μl of 2 x PCR reaction mix containing 10 x Taq buffer (2μl), magnesium chloride (2mmol), DNTPs (0.2mmol each) and Taq polymerase (2 units). Reactions proceeded in a thermal cycler for 40 cycles (denaturation 60 seconds 95°C, annealing 60 seconds 55°C, extension 60 seconds 72°C). PCR products were run on a 2% agarose gel containing ethidium bromide. Positive results were found as a band on gel electrophoresis corresponding to a 263bp product.

Detection of *REG* mRNA in Bone Marrow for Patients with Colorectal Cancer Was Determined As Follows:

17 consenting patients underwent bilateral bone marrow aspiration at the time of resectional surgery of a REG expressing colorectal cancer. Whilst patients with overt liver metastases diagnosed pre-operatively were excluded from the study, two patients were found to have previously undiagnosed metastatic disease at operation, and a further patient had incomplete excision of a locally invasive tumour. Pathological staging was available on 12 resected specimens and classified 1 as Dukes stage A, 5 as Dukes stage B and 6 as Dukes stage C disease.

Materials and Methods

Following the induction of anaesthesia, 3mm stab incisions were made over the sites of intended aspiration at the left and right posterior iliac crests to avoid epithelial cell contamination of the samples. 5ml of bone marrow was aspirated using a sterile, single-use needle and place in a heparinised container. Samples were then subjected to Ficoll™ density centrifugation. and the mononuclear layer was collected for further analysis. Following two

further washes in HBSS, the cell number in each sample was counted and cytospins were prepared at 10^6 total cells/ml, air dried and maintained at -20 C until required. $1x10^6$ total cells of the remaining sample were stored at -80 C until required for RNA analysis.

Immunocytochemistry

Defrosted slides were fixed in 70% ethanol for 10 minutes prior to immunostaining using the strepatavidin-biotin method with fast red as chromagen. Non-specific staining was minimised by initial pre-incubation with normal rabbit serum. Excess serum was removed and the primary anti-epithelial monoclonal antibody Ber-EP4 applied for 30 minutes at room temperature. Following washing and further rabbit serum blockade, a rabbit anti-mouse biotinylated conjugate was applied for 30 minutes(Dako UK). Following further washing and brief incubation with Tris buffer, alkaline phosphatase conjugated streptavidin was added for 30 minutes (Dako UK). After further washing, fast red solution containing levamisole (Dako UK) was added to each slide for 20 minutes. Following immersion in distilled water, slides were counterstained with Mayers haematoxylin and examined under light microscopy. Control positive slides were included in each batch of slides stained and for each pair of slides stained a further slide was subjected to the above protocol in the absence of a primary antibody to control for non-specific background staining. All slides were reviewed by an experienced consultant cytopathologist blinded to the RT-PCR experimental results.

RNA Extraction

Cell samples were suspended in 1ml of Catrimox™ cationic surfactant

solution on removal from -80 C storage. The solution was centrifuged for 5 minutes at 1000g, forming a detergent-bound RNA pellet. The supernatant was discarded, the RNA pellet resuspended in 1ml of 2M LiCl, and centrifuged at 13 000g for 5 minutes. A total of five cycles of washing and centrifugation in 2M LiCl were carried out, followed by a final wash with 70% ethanol at 4°C. Following centrifugation at 13 000g for 5 minutes the ethanol was discarded and the pellet dried in vacuum drying apparatus for 30 minutes. The RNA pellet was resuspended in 20μl of water containing 1mM dithiothreitol and 1U/μl RNAsin (Promega, Southampton).

RT-PCR

RNA (10μl) was reverse transcribed in a 20μl reaction using 120U MMLV-RT (Promega) 0.5μg oligo (dT), 1mM each dNTP 20U RNAsin , in 1X reverse transcription buffer with 3mM $MgCl_2$, at 42°C for 1 hour followed by a five minute heat inactivation period at 95°C. All cDNA samples underwent amplification using primers for a constitutively expressed gene (glyceraldehyde-3-phosphate dehydrogenase), to confirm RNA extraction and reverse transcription. Intron-spanning primers were devised to amplify a 263 base pair product of *reg* cDNA (Accession number Genbank/EMBL J05412) (*reg* upper dAACATGAATTCGGGCAACC, positions 2708....2726, exon 4; *reg* lower dGGCACATCCTTCCATTTCCT, positions 3880....3862, exon 6). Primers (1mM) and template (1 microlitre of RT reaction product) in a final volume of 10 μl were overlaid with mineral oil and heated to 95°C for 'hot start' PCR. The reaction was initiated by the addition of 10 μl of PCR reaction mix, to give Taq polymerase (1U)), dNTPs (0.2mM each, Pharmacia) 1X Taq buffer (Promega) and $MgCl_2$ (2mM). Reactions proceeded in a thermal cycler for 40 cycles (denaturation 60s 95°C, annealing

60s 55°C, extension 60s 72°C). PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and visualised by ultraviolet transillumination. Reg positivity was defined as the presence of a 263bp band.

Detection of *REG* mRNA in Normal-appearing Mucosa Around Colorectal Cancers

Existing data suggests that normal - appearing colorectal mucosa surrounding colorectal cancer is aberrant in its expression of glycoproteins and crypt architecture. In addition, recent data suggests that expression of growth-associated genes is significantly up-regulated in macroscopically normal tissue near the advancing margin of a colorectal cancer. We therefore investigated the expression of the human REG gene in epithelial tissues sampled from 6,4 and 2 cm proximal to a number of colorectal cancers.

Materials and Methods

Fresh mucosa specimens were obtained and snap frozen direct from surgical specimens at resection of histologically proven colorectal cancer. Samples were obtained 6,4 and 2 cm proximal to the tumour edge, in addition to tumour edge itself and the proximal resection margin of the surgical specimen, and stored at -80C. RNA extraction and RT-PCR were carried out as described above for bone marrow investigations in patients with colorectal cancer.

Results

31 tumour specimens had strongly positive bands indicating *REG* cDNA following the PCR reaction. 17 tumour specimens had very weak or absent bands. In four cases apparently normal mucosa samples from surgical resection specimens contained *REG* cDNA, but in each case only in association with a *REG* expressing tumour in the same specimen (Figure 1, Figure 2).

Examination of the Dukes' stage of tumours showed a trend towards *REG* expression in more advanced Dukes' C tumours whereas in earlier stage tumours there was no bias towards *REG* expression. In terms of gross survival, patients with *REG* expressing tumours, of all stages, showed a trend towards poorer cancer-specific survival with 13 out of 31 *REG* positive individuals dying from their cancer compared to only 2 out of 17 *REG* negative individuals.

Table 1

Dukes's stage	reg-positive (n=31)	reg-negative (n=17)
A	4	0
B	5	10
C	18	4
D	2	1
Recurrent/unknown	2	2

Bone Marrow Investigations

Dukes stage	Positive bone marrow samples (%)

A	0/1	(0%)
B	4/5	(80%)
C	1/6	(17%)
D (locally advanced)	0/1	(0%)
(distant metastases)	2/2	(100%)

Survival analysis

Survival analysis was carried out on the two groups, on all disease stages (Figure 6). Analysis of time to disease recurrence following surgery carried out with an expectation of cure was also carried out (Figure 7).

Normal-appearing mucosa investigations

Ectopic expression of the REG gene was identified in normal-appearing mucosa 2cm from REG expressing colorectal cancers in 10 of 13 cases. Expression of REG in both carcinomas and normal appearing tissue 2cm away was significantly more common than in tissue from the proximal resection margin or 6cm from the tumour edge (Figures 8 and 9).

Summary

These results demonstrate the ectopic expression of human *REG* in the majority of colorectal carcinomas. Analysis of normal mucosa samples from each resected specimen has shown four cases of *REG* expression in normal mucosa, each time in association with the tumour expressing this product. Clinical data has shown that patients with tumours expressing this gene are at significantly higher risk of early disease recurrence and death.

Control bone marrow samples obtained from large numbers of patients undergoing resectional abdominal surgery for benign disease were all consistently negative using this technique. Expression of REG mRNA in primary tumours indicates ectopic expression in tumour tissue and therefore expression in secondary deposits is likely. The presence of REG mRNA is therefore taken in this study to be indirect evidence of micrometastatic spread. Taken in conjunction with the above described results relating REG expression to prognosis in node-negative colorectal cancer, REG mRNA in bone marrow may add a further degree of accuracy to prognostication in colorectal cancer. It is also of interest that a high proportion of node-negative tumours expressing REG presented with REG mRNA present in bone marrow, supporting the view that REG expression may be a biomarker of aggressive behaviour in these tumours. Histopathological diagnosis of a 'node-negative' status may therefore be an unreliable indicator of lack of metastatic disease.

Moreover, our results agree with other investigator's who have shown occasional aberrant expression of the genes in normal-appearing mucosa up to 4cm from the advancing edge of a colorectal tumour. This is not likely to be due to infiltrating carcinoma as other studies have shown minimal microscopic spread of such tumours. The significance of these results in elucidating the pathogenesis of these tumours is not clear as an area of field change with REG expression may encourage tumour development, but equally, the effect of the tumour may induce REG expression in surrounding tissues. The abnormal expression of REG in tissue so far from the tumour edge has implications for surgical practice where the safety of surgical resection margins is of paramount importance. Furthermore, the expression of REG at the margins of surgical resection in these cases indicates aberrant

gene expression at the site of anastomosis. Prospective and retrospective investigations are needed to establish whether the growth promoting effects of REG expression at the site of colonic anastomosis predisposes to anastomotic recurrence.

PANCREATIC CANCER

Detection of *REG* mRNA in bone marrow from patients with pancreatic cancer

Materials and Methods

Bone marrow was aspirated from two sites in 15 consenting patients undergoing general anaesthesia with a diagnosis of pancreatic cancer based upon either cross sectional imaging using CT or MRI, or atypical cells obtained by brushing at ERCP or fine needle aspiration cytology. In addition, bone marrow was obtained from three patients with benign pancreatic pathology undergoing anaesthesia.

Bone Marrow Samples Were Processed As Previously Described for Bone Marrow Samples in Patients with Colorectal Cancer

Results

Immunocytochemistry was carried out on duplicate slides from 15 patients with histologically proven adenocarcinoma of the pancreas (10) and duodenum (2) and from a control group of four patients with benign pancreatic disease. In patients with proven carcinoma of the pancreatic head

or ampulla, tumour cells were detected in bone marrow by immunocytochemistry in one of 10 cases, and one of two duodenal carcinomas had bone marrow micrometastases. None of the four control cases had positively staining cells present.

Bone marrow from patients with pancreatic and ampullary tumours contained REG mRNA in 3 of 10 cases. This RT-PCR-positive group included the patient with positive marrow demonstrated by immunocytochemistry. The implication is that RT-PCR may well be more sensitive for the detection of micrometastases than microscopy-based techniques alone. No REG mRNA was present in the marrow of either patient with duodenal carcinoma or any of the non-malignant control cases.

Summary

The poor overall prognosis of pancreatic cancer is partially due to the frequently late presentation of the disease and hence advanced stage at the time when surgery is considered. Our group of pancreatic cancer patients consists solely of those patients with cross sectional imaging results suggesting that tumour resection might be possible, and this may explain why the rate of micrometastases detected by immunocytochemistry is somewhat lower than reported elsewhere . The presence of micrometastases (detected by histology) in the bone marrow of one of the cases of duodenal carcinoma was associated with an advanced tumour that required operative intervention for gastric outlet obstruction. This suggests that duodenal cancers may not usually express REG mRNA. Expression of REG mRNA in the bone marrow of the patient with positive marrow by immunocytochemistry, plus in two further cases suggests that REG RT-PCR may be a more sensitive

method of detection of small numbers of pancreatic cells in bone marrow than immunocytochemistry.

Detection of *REG* mRNA in Peritoneal Washings From Patients with Pancreatic Cancer

REG is constitutively expressed by the acinar cells, and in some malignant tumours of the ductal epithelium of the pancreas. The majority of pancreatic carcinomas arise from the ductal epithelium. We investigated the expression of REG mRNA from cells obtained from the peritoneal cavity at the time of surgery for apparently localised pancreatic carcinoma. REG mRNA was identified by RT-PCR, as previously described, in 40% of these cases. Potential applications of peritoneal disease status in pancreatic cancer revolve around the ability of viable tumour cells in the peritoneal cavity to predict early relapse following potentially curative surgery. The advantages of such prediction in pancreatic cancer would accrue from better targeting of an aggressive surgical approach. This may potentially reduce the number of aggressive procedures carried out on patients with a poor prognosis and limited survival potential following diagnosis in whom a palliative approach would be more appropriate. Additionally it may identify the cohort of patients in whom the investment of time and resources by both patient and surgeon in attempting cure would be fully justified.

PROSTATE CANCER

Detection of *REG* mRNA in Prostate Carcinoma

Materials and Methods

Samples of malignant prostate tissue were obtained from 10 patients undergoing trans-rectal ultrasound-guided biopsy of the prostate in an attempt to identify patients with previously undiagnosed prostrate cancer, and stored at -80 for subsequent RNA analysis. RNA was extracted and RT-PCR was carried out as previously described.

Results

3 of 10 samples expressed REG mRNA. The same size PCR product was obtained from prostate samples as had been obtained from colorectal cancer tissue and normal gastric mucosa. (Figure 10). Pathological examination by histological examination confirmed that these 3 of the 10 specimens did indeed contain malignant tissue.

Summary

Prostate cancer is a notoriously heterogeneous disease, with the majority of tumours being small and multifocal. Sampling error may therefore influence results based upon biopsy specimens leading to false negative results. The expression of the proliferation-associated gene REG in prostate cancer may be associated with the process of oncogenesis in this organ. Most intriguing is the clinical entity of benign prostatic hypertrophy (BPH), a very common condition in men, some of whom go on to develop invasive carcinoma of the prostate. REG expression could be used as a potential biomarker of malignant potential in BPH.

ULCERATIVE COLITIS

Detection of *REG* mRNA in Active and Quiescent Ulcerative Colitis

Both ulcerative colitis and Crohn's disease of the colon are known to predispose to carcinoma, and over-expression of human intestinal growth factors is known to be a feature of active inflammatory bowel disease. Rat models have shown increased expression of growth factors in inflammatory bowel disease models. Furthermore, expression of REG is locally induced in enterochromaffin-like cells in the gastric mucosa following the induction of stress ulcers. We have examined samples of mucosa obtained at colonoscopic biopsy from patients with inflammatory bowel disease in both the active and quiescent phases of their diseases to establish the pattern of REG expression in these inflammatory ulcerative conditions.

Materials and methods

Endoscopically obtained mucosal biopsies were snap frozen and stored at -80C until analysis from X patients with active inflammatory bowel disease. In all 16 patients, further endoscopic biopsies were obtained on later occasions at which time the naked eye appearance of the mucosa, as being either actively inflamed or in remission, was noted. Subsequent RNA extraction and RT-PCR was carried out as described above.

Results

11 of the 16 patients were REG positive by RT-PCR (Figure 6) only 3 of these remained positive in apparently quiescent disease.

Summary

The active phases of both Crohns disease and ulcerative colitis are characterised by regeneration in colonic epithelium in conjunction with ulceration and inflammation. The expression of REG appears to be linked to active disease status. Further work is ongoing to characterise REG expression in active inflammatory bowel disease. It is of interest that the risk of developing colorectal cancer is significantly increased in cases of IBD where inflammation is long-standing and widespread.

Conclusion

Our results have shown that *REG* gene expression is a common feature among colorectal carcinomas but it is a far less frequent feature of normal colonic mucosa, and then only in association with a local tumour. The presence of *REG* gene expression in association with *REG* expressing tumours raises the possibility of a field change surrounding the neoplasm. These results strongly support the use of *REG* gene expression as a means of identifying patients at the time of surgery who are at high risk of disease relapse. Such a feature may be used to select patients for the administration of adjuvant chemotherapy. In addition, *REG* expression status needs to be a strong predictor of clinical outcome such as this must be taken into account when planning and interpreting studies concerning the efficacy of adjuvant chemotherapy. Forthwith *REG* status should be established before accepting the similarity of two arms of randomised chemotherapy trials if bias is not to be introduced.

REG gene expression is also a common features of other cancers such as pancreatic cancer where, again, the accurate prediction of post-operative outcome is a worth while end and the place of immunocytochemistry in

refining this has been demonstrated in numerous tumours of the gastrointestinal tract. Increases in sensitivity using methodology such as RT-PCR offer the possibility of further identifying those patients most at risk of treatment failure, identification of which will have important implications in the evaluation and implementation for adjuvant therapy for pancreatic cancer. This is also true of prostrate cancer where *REG* expression can be used as a marker for malignant tissue. Additionally, *REG* expression can also be used to predict a tendency towards the development of malignancy in diseased conditions, such as ulcerative colitis, which are known to progress in this manner. In conclusion, the assay of *REG* gene expression may be of general utility in assessing prognosis in cancer patients at presentation and in guiding the choice of aggressive or less potentially toxic treatment modalities.

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CLAIMS

1. An assay system for identifying the existence of a product or products of the pancreatic protein lithostaphine (*REG*) gene, so as to determine a tendency to develop cancer or the aggressive nature of an existing cancer, comprising; means for detecting said product or products.

2. An assay system according to Claim 1 wherein said means involves material suitable for extracting mRNA encoded by said gene from a sample solution or tissue.

3. An assay system according to Claim 2 wherein said means further includes material suitable for performing reverse transcription of said mRNA.

4. An assay system according to Claims 1, 2 or 3 wherein said means further includes primers devised to amplify a selected part of said *REG* gene.

5. An assay system according to Claim 4 wherein said primers are adapted to hybridise with at least an intron part of said gene.

6. An assay system according to Claim 5 wherein said primers are adapted to amplify between exon 4 and exon 6 of said gene.

7. An assay system according to Claim 1 wherein said means comprises antibodies adapted to bind to at least a part of a *REG* gene product.

8. An assay system according to Claim 7 wherein said antibodies are monoclonal.

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9. An assay system according to Claim 7 or 8 wherein further antibodies may be provided in order to amplify the signal provided by the reaction.

10. An assay system according to Claims 7, 8 or 9 wherein said antibodies may be provided with suitable labels in order that the binding of same to at least one *REG* gene product may be determined.

11. A screening procedure for determining the efficacy of a selected therapeutic comprising:

a) examining cancer tissue and/or fluid associated with same from an individual who may take part in said screening in order to determine whether *REG* gene is transcribed and/or translated; and

b) depending upon the results of a) above including or excluding said individual from trials to investigate the efficacy of said therapeutic.

12. The use of a *REG* gene product for the determination of a tendency towards a cancerous condition or the aggressive nature of cancer tissue.

13. A method for determining a tendency towards a cancerous condition or the aggressive nature of cancer tissue comprising examining, respectively, tissue or associated fluid, likely to become cancerous, or said cancer tissue and/or fluid associated with same to identify a product of *REG* gene transcription and/or translation.

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14. A kit for use in the methods of any proceeding claim which kit comprises at least one nucleic acid probe and/or primer and instructions for the use.

Figure 1

In 31 tumours *reg* mRNA was strongly expressed

17 tumours showed no *reg* expression

Dukes's stage

	reg-positive (n=31)	*reg* negative (n=17)
Dukes' A	4	0
Dukes' B	5	10
Dukes' C	18	4
Dukes' D	2	1
Unknown/Recurrent	2	2

Figure 2

Patients undergoing potentially curative surgery for reg positive tumours are at increased risk of developing disease recurrence within two years.

	reg-positive tumours	reg-negative tumours
Tumour recurrence	13/29	1/16

$p < 0.05$ Logrank test

Figure 3

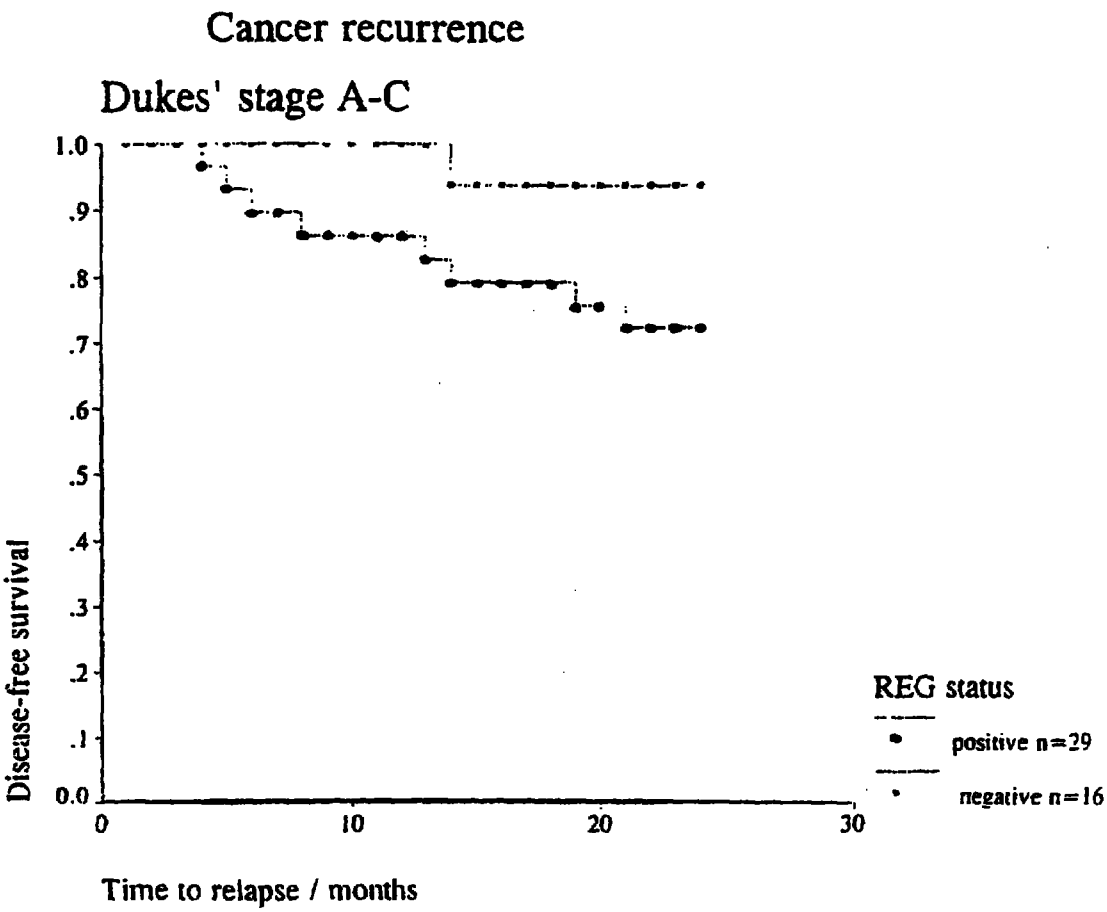

Cancer recurrence
Dukes' stage A-C
Disease-free survival
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
0
10
20
30
Time to relapse / months
REG status
positive n=29
negative n=16

Figure 4

Patients with *reg* expressing tumours undergoing potentially curative surgery showed a significantly increased incidence of cancer related death over the two year follow up period

	reg-positive tumours	*reg*-negative tumours
Cancer related deaths	11/29	1/16

$p < 0.05$ Logrank test

Figure 5

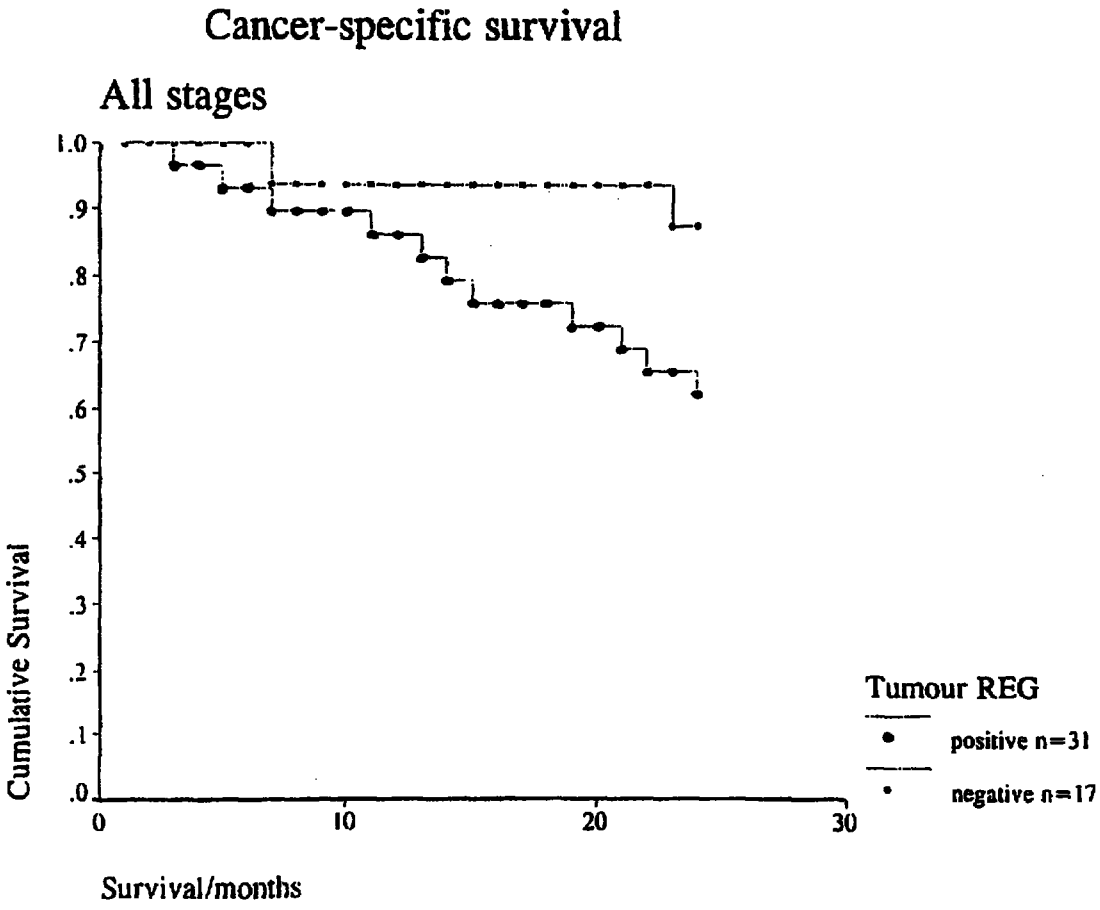

Cancer-specific survival
All stages
Cumulative Survival
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
0
10
20
30
Survival/months
Tumour REG
positive n=31
negative n=17

Figure 6

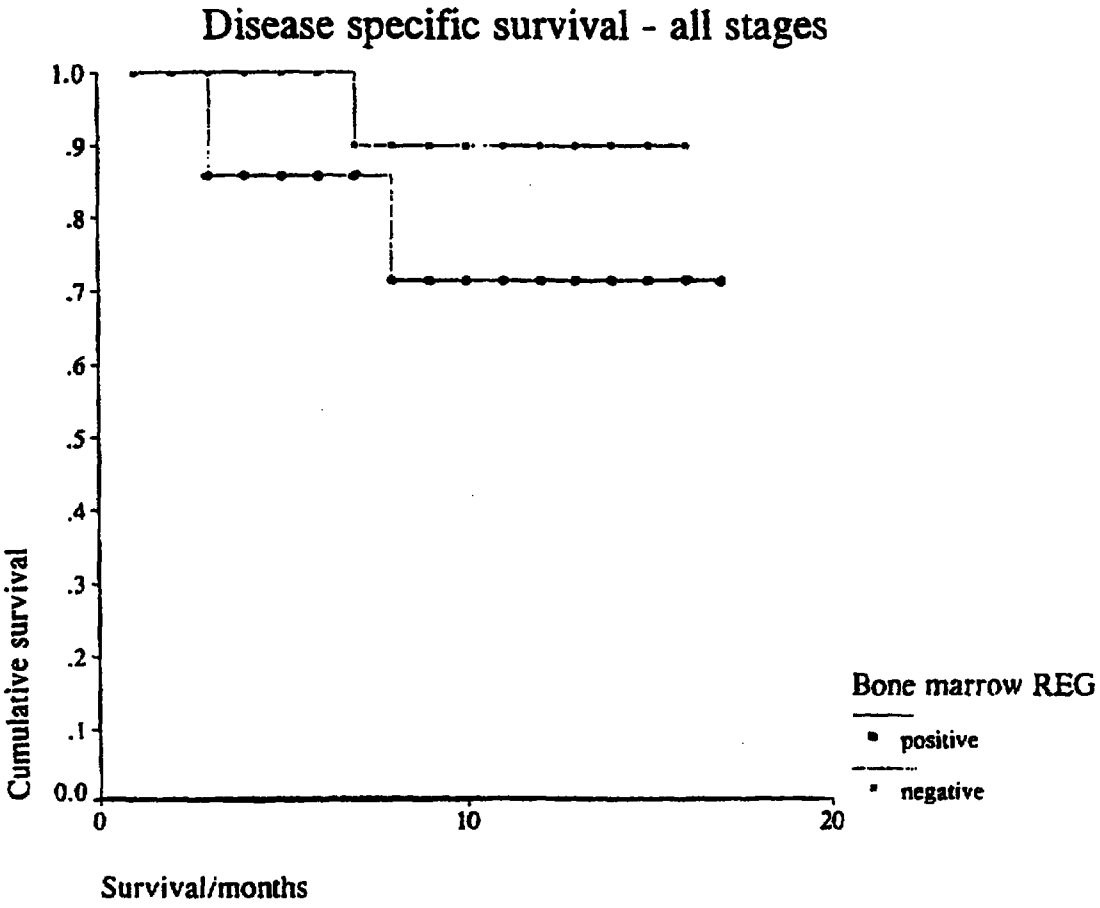

Disease specific survival - all stages
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
Cumulative survival
0
10
20
Survival/months
Bone marrow REG
positive
negative

Figure 7

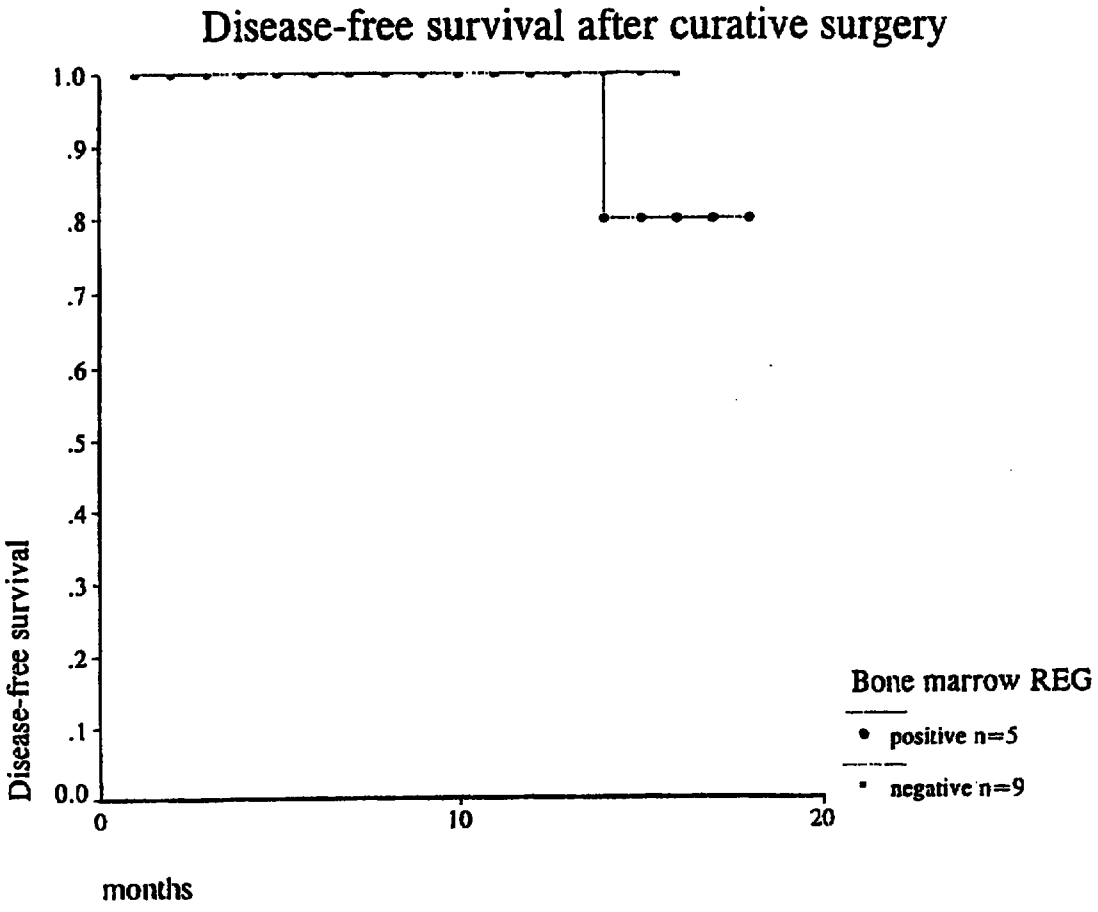

Disease-free survival after curative surgery
Disease-free survival
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
0
10
20
months
Bone marrow REG
positive n=5
negative n=9

Figure 8

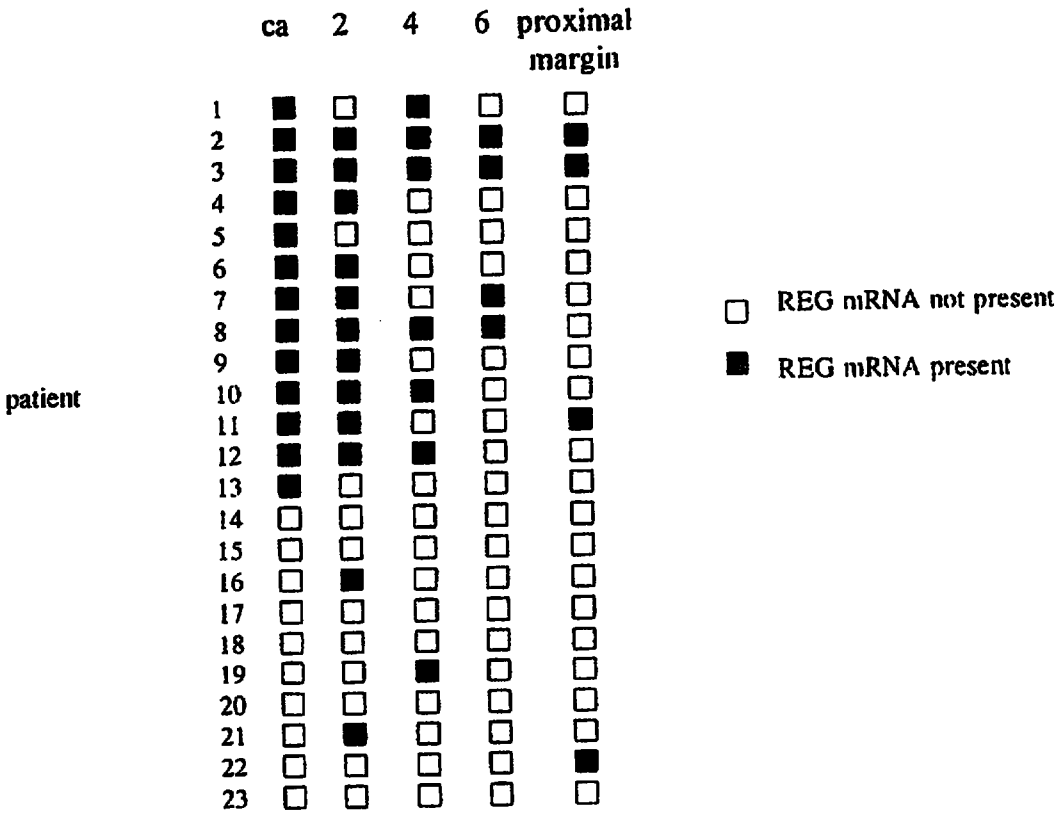

ca
2
4
6
proximal margin
patient
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
REG mRNA not present
REG mRNA present

Figure 9

	carcinoma	2cm	4cm	6cm	margin
reg expressing samples	13* (57%)	12** (52%)	7 (30%)	4 (17%)	4 (17%)

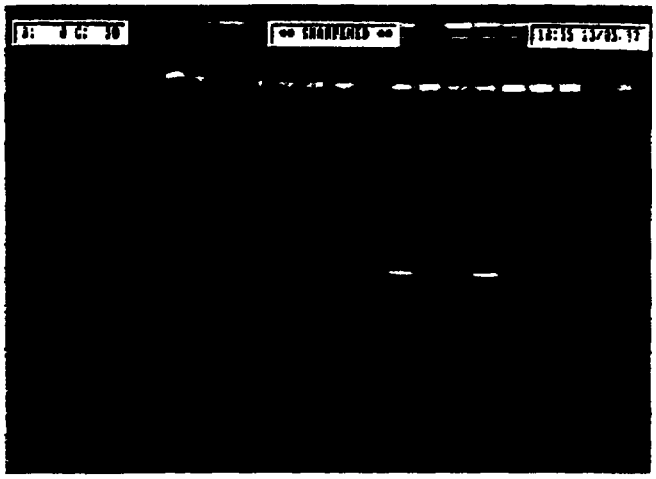
*p=0.02 carcinoma vs distal margin and 6cm (chi square with Yates correction)

**p=0.03 2cm vs distal margin and 6cm (chi square with Yates correction)

Figure 10

PCR REACTION 2

Lane 1	Patient 1	Negative
Lane 2	Patient 2	Negative
Lane 3	Patient 3	Negative
Lane 4	Patient 4	Negative
Lane 5	Patient 5	Positive
Lane 6	Patient 6	Negative
Lane 7	Patient 7	Positive
Lane 8	Patient 8	Negative
Lane 9	Patient 9	Positive
Lane 10	Patient 10	Negative
Lane 11	Neg Control	Negative
Lane 12	Pos Control	Strong positive



PCR REACTION 1

Lane 1	Patient 1	Negative
Lane 2	Patient 2	Negative
Lane 3	Patient 3	Negative
Lane 4	Patient 4	Negative
Lane 5	Patient 5	Negative
Lane 6	Patient 6	Negative
Lane 7	Patient 7	Positive
Lane 8	Patient 8	Negative
Lane 9	Patient 9	Positive
Lane 10	Patient 10	Negative
Lane 11	Neg Control	Negative
Lane 12	Pos Control	Weak positive

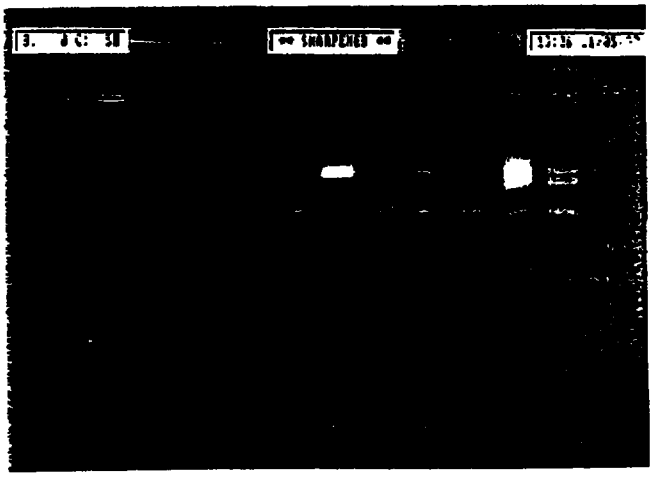
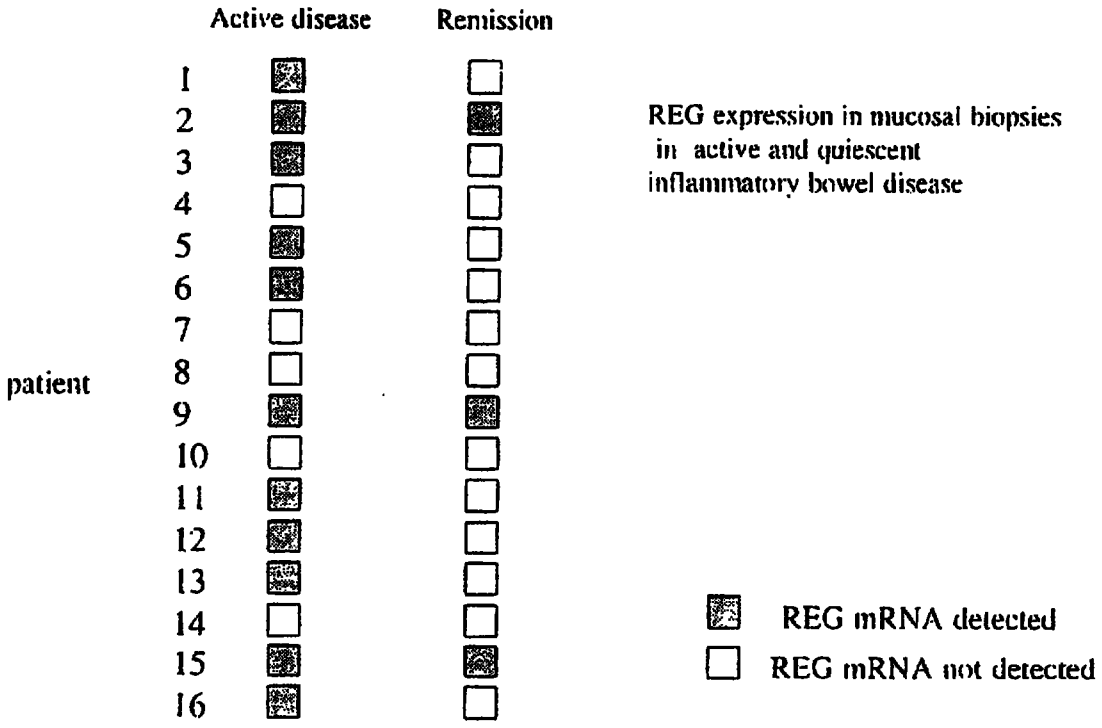


Figure 11


Active disease
Remission
patient
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
REG expression in mucosal biopsies in active and quiescent inflammatory bowel disease
REG mRNA detected
REG mRNA not detected

REPLACEMENT SHEET

0 1 DEC 1999

CLAIMS

1. A method for determining a tendency to develop cancer or determining the aggressive nature of an existing cancer in an individual comprising, detecting a product or products of the human *REG* gene in a sample from said individual.

2. A method according to Claim 1 wherein the product of the human REG gene is REG mRNA.

3. A method according to Claims 1 or 2 which involves the use of primers to amplify a selected part of said *REG* gene.

4. A method according to Claim 3 wherein said primers are adapted to hybridise with at least an intron part of said gene.

5. A method according to Claim 4 wherein said primers are adapted to amplify between exon 4 and exon 6 of said gene.

6. A method according to Claim 1 wherein REG gene product is determined by binding of antibodies to at least a part of a *REG* gene product.

7. A method according to Claim 6 wherein said antibodies are monoclonal.

8. A method according to Claim 6 or 7 wherein further antibodies are used in order to amplify the signal provided by the reaction.

9. A method according to Claims 6, 7 or 8 wherein said antibodies may be provided with suitable labels in order that the binding of same to at least one *REG* gene product may be determined.

10. A screening procedure for determining the efficacy of a selected therapeutic comprising:

REPLACEMENT SHEET
0 1 DEC 1999

a) examining cancer tissue and/or fluid associated with same from an individual who may take part in said screening in order to determine whether *REG* gene is transcribed and/or translated; and

b) depending upon the results of a) above including or excluding said individual from trials to investigate the efficacy of said therapeutic.

11. The use of a *REG* gene product for the determination of a tendency towards a cancerous condition or the aggressive nature or cancer tissue.

12. Use of *REG* gene expression as a biomarker of malignant potential in benign prostatic hypertrophy.

13. Use of *REG* gene expression to predict a tendency towards malignancy in individuals suffering from ulcerative colitis.

14. Use of *REG* gene expression for predicting post operative outcome.

15. A method for determining a tendency towards a cancerous condition or the aggressive nature of cancer tissue comprising examining, respectively, tissue or associated fluid, likely to become cancerous, or said cancer tissue and/or fluid associated with same to identify a product of *REG* gene transcription and/or translation.

16. A method for predicting the likelihood of micrometastatic spread in a cancer patient comprising detecting the presence of *REG* mRNA in primary tumour tissue.

17. A kit for use in a method according to any of claims 1 - 10, 15 or 16, which kit comprises at least one nucleic acid probe and/or primer and instructions for the use.

18. A kit for use in a method according to any of claims 1 - 10, 15 or 16, which kit comprises at least one antibody and instructions for the use.

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(57) Abstract

The invention relates to a marker for determining the tendency towards a cancerous condition and/or the aggressive nature of cancer tissue with a view to determining a prognosis and suitable course of action/treatment. More specifically, the invention concerns an investigation for products of the (*REG*) gene, i.e. the pancreatic protein lithostathine (*REG*) gene.