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Effect of essential fatty acids on circulating T cell subsets in patients with colorectal cancer

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Abstract. The effect of essential fatty acids (EFA), given orally as dietary supplements, on the responsiveness in vitro of peripheral blood lymphocytes (PBL), to the mitogen concanavalin A have been studied in 10 patients with localized and 14 patients with advanced colorectal cancer. The degree of lymphocyte activation was assessed by measuring the amount of tritiated [^{3}H]thymidine incorporated into newly synthesised lymphocyte DNA. The results were expressed as stimulation indices. T cell responses to concanavalin A stimulation showed a significant reduction of stimulation indices following EFA supplementation, in both the localized (P = 0.026) and advanced (P = 0.016) tumour groups, when compared with pretreatment activity in vitro. Mixing experiments, using EFA-supplemented and non-EFA-supplemented lymphocytes with concanavalin A, suggest no enhancement of T suppressor cell activity. Cell surface marker analysis (fluorescence-activated cell sorting for CD phenotyping) revealed a reduction of absolute numbers of CD4$^+$ and CD8$^+$ lymphocytes following EFA supplementation. The stimulation indices returned to pre-supplementation values 3 months following cessation of EFA intake. There was no significant change of these indices in the control (no EFA supplementation) advanced tumour group tested. This study suggests that EFA supplementation in patients with colorectal cancer selectively reduces circulating PBL, and T cell subset (including suppressor cells) numbers and/or activity. Such effects may have an important outcome in patients with malignant disease.

Key words: Essential fatty acids – Cell proliferation – Colorectal cancer

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Introduction

A number of published reports support the concept that essential fatty acids (EFA) may play a role in regulating immune mechanisms [9, 15–17, 22]. First, lymphocyte stimulation by mitogens or antigens in vitro alters the fatty acid composition of the membrane [7, 12]. Second, alterations of dietary fats in rodents are reflected in changes in the fatty acid composition of lymphocytes [22]. Moreover, addition of EFA to lymphocytes in vitro has been shown to modulate various immune functions [9, 11, 13, 17, 22, 32]. However, responses after manipulation of fatty acids in vitro may not reflect immune changes in vivo, because of the complexity of cellular and humoral interactions in vivo. Human T lymphocytes stimulated in vitro by the mitogen, concanavalin A (ConA), generate various functionally distinct and expanded T cell populations, e.g. cytotoxic T cells, T helper cells, and inhibitors of immune reactivity (suppressor cells) [8, 27]. The activation of suppressor cell precursors or promotors leads, in turn, to inhibition of both B and T cell functions.

Scotland has one of the highest incidences world-wide of carcinoma of the colon and a high death rate from the disease. In an attempt to improve the latter, attention has focused on new forms of systemic therapy, in particular, on the ability of EFA both to destroy malignant cells and to modulate anticancer immune defence mechanisms. Essential fatty acids (n-3 and n-6) have been shown to be toxic in vitro to human breast, lung and prostate cancer cell lines whilst leaving normal human fibroblasts and other normal cells unaffected [2]. Several reports have documented that dietary supplementation with plant oils rich in n-6 polyunsaturated fatty acids and fish oils (rich in n-3 polyunsaturated fatty acids) improved the condition of patients suffering from benign diseases such as rheumatoid arthritis [19], multiple sclerosis [29] and psoriasis [4].

To the best of our knowledge, there have been no reports on the effects of a high concentration of EFA in vivo on the in vitro lymphocyte reactivity of peripheral blood lymphocytes (PBL), obtained from patients with colorectal cancer. In this study, we document the effects of EFA, given orally

as dietary supplementation, on the responsiveness of PBL to the mitogen ConA in vitro, in patients with clinically localized and advanced colorectal cancer. Furthermore, the lymphocyte subsets of PBL in such patients were evaluated, using various CD antibodies and a fluorescence activated cell sorter (FACS). Our data suggest selective reduction of T suppressor cell subsets with EFA supplementation. This may have important implications for patients with malignant disease.

Materials and methods

Clinical material. Patients, male and female, aged 46–86 years (mean 63.8 $\pm$ 2.33), with clinically localized (n = 10) and advanced (n = 20) colorectal cancer, were studied. Patients were staged by clinical examination, blood tests (full blood count, electrolytes and urea, liver function tests, serum carcinoembryonic antigen) and various imaging modalities (ultrasound, computed tomography scanning, barium studies, etc.). Where appropriate, tissue diagnosis was obtained prior to surgery. The patients were divided into three groups as outlined below.

1. Patients with clinically localized colorectal cancer, received EFA (HGD6-Scotia) supplements (four capsules twice a day) orally for 15 days prior to surgical treatment. EFA supplements were discontinued following surgical excision of the tumour.

2. Patients with advanced colorectal tumours (e.g. extensive hepatic metastases, irresectable intra-abdominal disease) received EFA (HGD6-Scotia) supplements orally in increasing doses (four capsules twice daily for 15 days then six capsules twice daily for the next 15 days and thereafter eight capsules twice daily) without surgical intervention for 6 months.

3. Patients with advanced colorectal cancers received no EFA supplementation for 6 months without surgical intervention.

All patients involved in this study received no other medication or treatment during this investigation.

Venous blood was donated by both patients and healthy adult volunteers. Lymphocytes (40 ml) and serum (10 ml) were obtained from the venous blood of patients at different times (days 0 and 15; months 1, 2, 3, 4, 5 and 6; month 3 following cessation of EFA supplementation).

Essential fatty acids (HGD6-Scotia) were supplied from Scotia Pharmaceuticals Ltd. as enteric coated, oblong hard gel capsules containing 146 mg γ-linolenic acid, 20 mg docosahexaenoic acid, 132 mg eicosapentaenoic acid, 34.8 mg lithium and 0.25 mg ascorbyl palmitate. Some of the γ-linolenic acid was in the form of the lithium salt, which is partially water-soluble and may assist in the absorption and distribution of fatty acids. The amount of EFA ingested by each patient was calculated as shown in Table 1.

Fatty acid uptake and the patient compliance were confirmed by a significant increase in percentage of serum γ-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid in all three fractions: triglyceride, cholesterol and phospholipids following EFA supplementation (data not shown).

Approval for the study was given by the Joint Ethical Committee of the University of Aberdeen and the Grampian Health Board.

Table 1. The amount of essential fatty acids ingested by patients at different times

Administration schedule	GLA (mg)	EPA (mg)	DHA (mg)	Total (g)
Capsule	146	132	20	0.298
Daily dose 0–15 days	1168	1056	160	2.384
Daily dose 16–30 days	1752	1584	240	3.576
Daily dose >30 days	2336	2112	320	4.768

GLA, γ-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

Reagents used. Concanavalin A was obtained from Sigma Chemical Company, Poole, Dorset, England. Fetal calf serum (FCS) and HEPES-buffered RPMI-1640 medium were obtained from Life Technologies Limited, Paisley, Glasgow, Scotland. Disposable multiple-well plates (96 wells, round-bottomed) were obtained from Corning Bibby Sterilin Ltd., Staffordshire, England. Ficoll/Hypaque reagent for isolation of human blood lymphocytes (density 1.077 g/cm^3) was obtained from Pharmacia LKB Limited, Milton Keynes, England. [^{3}H]Thymidine was obtained from Amersham International, Amersham, England, and scintillant alkyl aromatic solvent (Optiscint) from Pharmacia LKB Limited, Milton Keynes, England. Phosphate-buffered saline (PBS), containing goat serum, was made up to standard 5% and 1% formulation. Goat serum (heat-inactivated at 56 °C for 1 h), 0.1% sodium azide, and mouse monoclonal antibodies reactive with lymphocyte surface marker antigens (CD2, CD3, CD4, CD8, CD19, CD22, CD25) were from Scottish Antibody Production Unit, Lanark, Scotland, Becton Dickinson, Oxford, England, and DAKO, High Wycombe, England. Goat anti-(mouse Ig) fluorescein-isothiocyanate(FITC)-conjugated serum was obtained from Becton Dickinson, Oxford, England.

Lymphocyte preparation. PBL were isolated from heparinised venous blood using differential centrifugation (400 g for 40 min) on a density gradient of Ficoll/Hypaque and washed in tissue-culture medium RPMI-1640. This culture medium contained 100 U penicillin/ml, 100 mg streptomycin/ml, 0.7 g sodium bicarbonate/l and 25 mM HEPES/l and 10% heat-inactivated FCS.

Mitogen assays. Lymphocyte suspensions were prepared at 1.25×10^6 cells/ml in RPMI medium containing 2 g/l sodium bicarbonate, 10% heat-inactivated FCS, 100 U penicillin/ml, 100 mg streptomycin/ml and 25 mM HEPES buffer/l.

Lymphocytes were cultured in the wells of a microtitre culture plate (80 µl cells/well) with 80 µl ConA at different concentrations (8, 32, 125, 250, 500 µg/ml). The plate was then incubated at 37 °C in a 5% CO_2 incubator for 48 h.

A 25 µl sample of [^{3}H]thymidine (37 kBq, 1 µCi) was then added to each well and the plate incubated for a further 18 h. Cells were harvested onto glass-fibre filters, which were washed and dried at room temperature for 24 h. The glass-fibre discs were then placed into 2 ml vials, 2 ml scintillant (Optiscint) was added to each vial and radioactive [^{3}H]thymidine incorporation measured in a β counter. The results are expressed as a stimulation index (SI), which was calculated as follows:

$$SI = \frac{[^3H]\text{thymidine incorporation in the presence of ConA}}{[^3H]\text{thymidine incorporation in the absence of ConA}}$$

Lymphocyte surface marker assays: flow cytometry. Lymphocytes in RPMI-1640 medium with 10% heat-inactivated FCS, at 1×10^7 cells/ml, were incubated with mouse monoclonal antibodies raised against various CD antigens in PBS containing 5% heat-inactivated goat serum and 0.1% sodium azide for 40 min in melting ice. The cells were washed with PBS containing 1% heat-inactivated goat serum and 0.1% sodium azide. A 100 µl sample of FITC-labelled goat anti-(mouse Ig) was added and the cells were incubated for a further 40 min in melting ice. Following this incubation period, 0.5 ml PBS containing 0.1% sodium azide was added and the sample analysed by flow cytometry (Coulter EPICS profile II).

The results obtained from flow cytometry were the percentage of positive cells (PC) within a defined lymphocyte bitmap and the mean channel as a measure of their intensity. The absolute number of positive cells was then calculated as follows:

$$\text{absolute number} = \frac{\text{PC (\%)} \times \text{total lymphocyte count (l}^{-1}\text{)}}{100}$$

Results

Effect of EFA in vivo on ConA-stimulated proliferation of PBL in vitro in patients with colorectal cancer

Localized colorectal cancer group (n = 10). T cell responses to ConA stimulation at day 15 following ingestion of EFA showed a significant reduction of SI, when compared with day 0 (Fig. 1), with a *P* value of 0.026 (paired *t*-test). The mean SI ±SEM for the 10 patients before and after in vivo EFA supplementation was 30.87±7.37 and 19.44±3.37 respectively, as shown in Fig. 1.
Advanced colorectal cancer group (n = 14). T cell responses to ConA stimulation showed a significant reduction of SI following EFA supplementation, when compared with day 0 (Fig. 1), with a *P* value below 0.05 (Fig. 1). The reduction began at day 15 after EFA intake and values continued to fall, reaching a significant minimal level at 6 months (Fig. 1). The mean SI ±SEM for the 14 patients before and during EFA supplementation is shown in Fig. 1. Mixed lymphocyte cultures of mitogen-stimulated lymphocytes (EFA-treated and healthy volunteers) were also carried out in order to determine whether the reduction of SI was due to suppression of lymphocyte proliferation rather than stimulation of suppressor cells, which in turn inhibited lymphocyte proliferation. The results of the mixed lymphocyte cultures suggested that the reduced SI in EFA-supplemented PBL was due to inhibition of lymphocyte proliferation (Fig. 2). The amount of tritiated thymidine incorporated into newly synthesized DNA indicated an increased cell proliferation when non-EFA-treated lymphocytes were added. The pattern of overall responses was determined by the various lymphocyte mixing ratios.

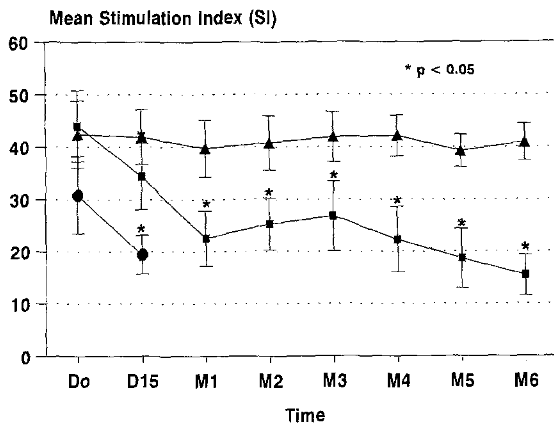

Fig. 1. Stimulation index of concanavalin-A(ConA)-stimulated peripheral blood lymphocytes (PBL) in patients with advanced colorectal cancer during essential falty acid (EFA) supplementation (*n* = 14; ■) and without EFA supplementation (*n* = 6; ▲), and patients with localized cancer (*n* = 10; ●) studied at various times: days (*D*) 0, 15; months (*M*) 1, 2, 3, 4, 5, 6

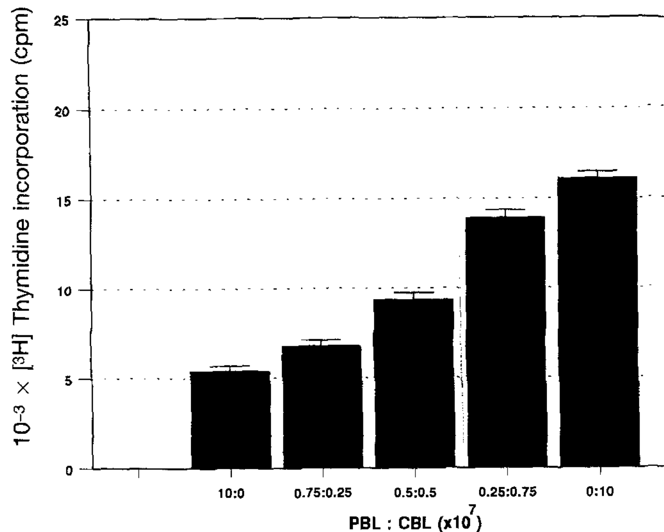

Fig. 2. Effect on in vitro ConA-stimulated lymphocytes, obtained from patients receiving EFA (PBL) and healthy controls (*CBL*), mixed at various PBL:CBL ratios (*n* = 6). The dose of ConA used in this experiment is 8 μg/ml. The ordinate shows $10^{-3} \times [^3H]$thymidine incorporation (cpm)

PBL proliferation in avdanced colorectal cancer patients (controls) without EFA supplementation (n = 6)

Six patients with advanced colorectal cancer without EFA supplementation were tested for proliferative responses using the mitogen ConA. There was no alteration in SI of ConA-stimulated PBL during the 6-month period tested. The mean SI values ±SEM are shown in Fig. 1.

Effect of ConA-stimulated PBL proliferation after cessation of EFA supplementation (n = 6)

There were 6 patients, in the advanced colorectal cancer group, available for follow-up after cessation of EFA supplementation. The T cell response to ConA stimulation showed an increase in SI, when evaluated 3 months after EFA cessation. The SI values returned to pre-supplementation levels in all 6 patients as shown in Fig. 3.

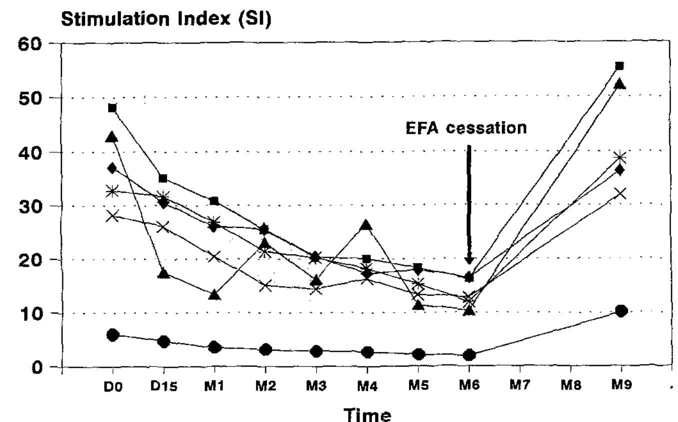

Fig. 3. Stimulation index (*SI*) of ConA-stimulated PBL in patients with advanced colorectal cancer before, during and 3 months after cessation of EFA supplementation (*n* = 6). The *symbols* (■, ●, *, ▲, ×, ◆) refer to each individual patient, studied at various times: days (*D*) 0, 15; months (*M*) 1, 2, 3, 4, 5, 6

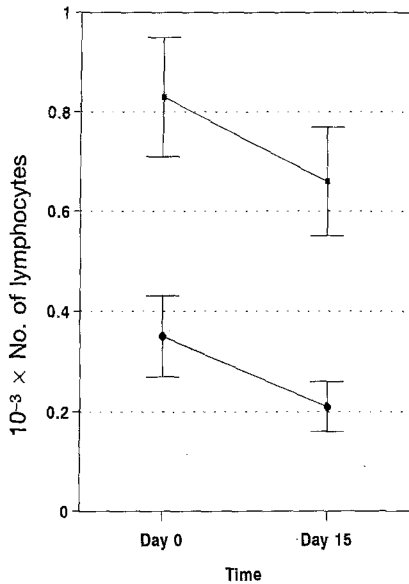

Fig. 4. Mean absolute number of CD4 (■) and CD8 (◆) lymphocytes during EFA supplementation in patients with localized colorectal cancer (n = 10). The values were determined at day 0 and day 15

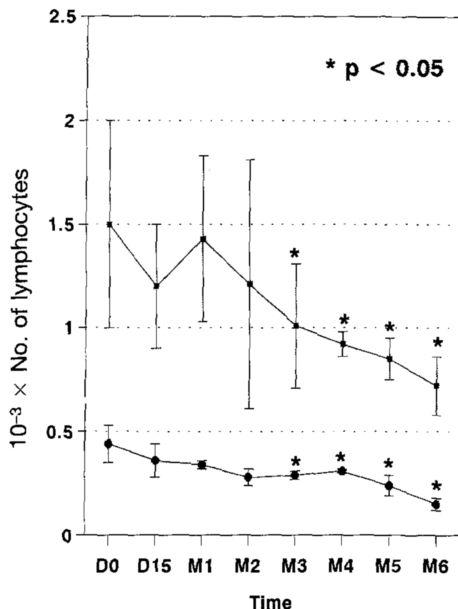

Fig. 5. Mean absolute number of CD4 (■) and 8 (●) lymphocytes during EFA supplementation in patients with advanced colorectal cancer (n = 14). The values were determined at various times: days (*D*) 0, 15; months (*M*) 1, 2, 3, 4, 5, 6

Effect of in vivo EFA on circulating lymphocyte subsets in patients with colorectal cancer

Cell-surface membrane CD antigens were analysed in patients with clinically localized and advanced colorectal cancers following dietary EFA supplementation. There was a marked reduction of the absolute number of CD4+ CD8+ lymphocytes in the localized tumour group at day 15, when compared with day 0 (prior to EFA supplementation; Fig. 4) (P = 0.050 and 0.036; paired t-test). A significant reduction of CD4+ and CD8+ lymphocytes was also shown after prolonged EFA intake in the advanced tumour group and minimal levels were reached at 6 months (Fig. 5) (P <0.05). There was a trend for the other CD subsets (CD2, CD3) in both the localized and advanced tumour groups to be reduced in the absolute number of cells but this was not statistically significant (data not shown).

Discussion

This present study has shown that EFA (γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid), given as a combined dietary supplement to patients with colorectal cancer, both localized and advanced tumours, inhibited [^{3}H]thymidine incorporation into ConA-stimulated PBL in vitro. The longer the EFA intake, the greater the suppression. The inhibitory effect of EFA upon lymphocyte proliferation in the advanced group was abolished after cessation of EFA intake, as shown by a return of SI (assessed at 3 months) to the pre-supplementation values (Fig. 3). Furthermore, there was no alteration in SI, in the 6-month period tested, in the non-EFA-supplemented patients with comparable advanced disease (Fig. 1). Cell-mixing experiments, with lymphocytes from donors not ingesting EFA supplements, indirectly demonstrated that the low levels in immune reactivity to ConA in vitro were not due to enhanced production of T suppressor cells (Fig. 2). The significant reduction of blood lymphocyte SI values, following EFA administration, was associated with a concurrent significant reduction of the CD4+ CD8+ T lymphocyte subsets (Figs. 4, 5). Our findings suggest that ingestion of EFA rapidly suppresses the in vitro proliferation of mitogen-stimulated T lymphocytes, including suppressor cells, in patients with both localized and advanced colorectal cancer. The data obtained suggest that the reduction in SI was not due to disease progression but was probably due to the influence of EFA upon circulating T cell subsets. There are several possible mechanisms to explain how the exogenous EFA may exert their inhibitory effects.

First, as fatty acids are components of phospholipids, the nature of the intracellular fatty acid pool may influence the degree of saturation of fatty acids in the phospholipids of cell membranes, and this could result in a change in membrane fluidity [1, 7, 26, 28]. It is possible that the altered membrane fluidity could cause inhibition of one or more of the membrane functions essential to the proliferation process. Such changes in membrane composition, if they occurred in this study, would cause the observed decrease in lymphocyte proliferation as indicated by the low SI following EFA administration.

A second possible mechanism by which fatty acids might inhibit lymphocyte proliferation may be via production of prostaglandins and/or other cyclooxygenase or lipoxygenase products [6, 23, 33]. There is evidence that prostaglandins inhibit lymphocyte responses. The E series of prostaglandins (PGE1, PGE2) have been shown to inhibit phytohaemagglutinin-induced T lymphocyte proliferation, T-cell-mediated cytotoxicity and lymphocyte-mediated allogeneic cell destruction in vitro, and inhibit graft rejection [14, 16, 18, 22], in vivo. Mihas et al. [24] found that the synthetic prostaglandin E2 caused a concentration-dependent suppression of [^{3}H]thymidine uptake

by lymphocytes stimulated with phytohaemagglutinin, but the inhibition was significantly less than that caused by oleic, linoleic and arachidonic acids, suggesting that conversion of the unsaturated fatty acids to prostaglandins is not the sole mechanism of immune suppression and/or that EFA themselves may exert a direct inhibitory effect [10, 24]. There have been many other studies showing that PGE1 and PGE2 inhibit T cell functions [14] but there is great variability in the suppression documented.

An effect of other fatty-acid-derived metabolites cannot be excluded. Certain cyclooxygenase-derived products (thromboxanes) and lipoxygenase-derived products (leukotrienes, hydroxyeicosatetraenoic acids, hydroperoxyeicosatetraenoic acids) are known to effect mitogen-induced lymphocyte proliferation [14, 16]. It will be of interest to determine the concentration of such compounds in the serum of these patients to establish whether there is a correlation with the effects of EFA on lymphocyte proliferation.

An important possible mechanism by which EFA could inhibit lymphocyte proliferation is by direct interference with the signal transduction mechanism [31]. Cytokines play a key immunoregulatory role. Interleukin-1 (IL-1) is an important early signal from monocytes/macrophages to T helper lymphocytes to produce IL-2, which then upregulates the production and secretion of IL-2 in an autocrine manner. The latter induces the expression of IL-2 receptors (α and β) and enhanced interaction with IL-2 on various lymphocytes subsets. These changes lead to increased transmembrane signal transduction (with resultant activation of intracytoplasmic metabolic pathways) and IL-2 translocation (with resultant gene regulation and cell proliferation). This process is modulated by IL-2 and IL-4 through their activity on T helper cells and by tumour necrosis factor α through activation of monocytes/macrophages and release of IL-1. Another possibility is that an increase in intracellular concentration of EFA could stimulate the rate of the triacylglycerol/fatty acid substrate cycle [3] leading to depletion of intracellular ATP levels.

Whatever the mechanism(s) involved in the inhibition of lymphocytic reactivity and thymidine incorporation by EFA, our results are consistent with the suggestion that dietary EFA may be selectively immunosuppressive, especially if given in high doses for a prolonged period of time. Although such inhibition of lymphocytic function could be detrimental, inhibition of T suppressor cell activity may be beneficial, particularly in patients with malignant disease. In the latter, excess activity of suppressor mechanisms has been documented and postulated to be responsible, in part at least, for progression of tumour growth [20, 21, 25, 30].

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