High Tumour Cannabinoid CB₁ Receptor Immunoreactivity Negatively Impacts Disease-Specific Survival in Stage II Microsatellite Stable Colorectal Cancer

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Abstract

Background: There is good evidence in the literature that the cannabinoid system is disturbed in colorectal cancer. In the present study, we have investigated whether CB₁ receptor immunoreactive intensity (CB₁IR intensity) is associated with disease severity and outcome.

Methodology/Principal Findings: CB₁IR was assessed in formalin-fixed, paraffin-embedded specimens collected with a consecutive intent during primary tumour surgical resection from a series of cases diagnosed with colorectal cancer. Tumour centre (n = 483) and invasive front (n = 486) CB₁IR was scored from 0 (absent) to 3 (intense staining) and the data was analysed as a median split i.e. CB₁IR <2 and ≥2. In microsatellite stable, but not microsatellite unstable tumours (as adjudged on the basis of immunohistochemical determination of four mismatch repair proteins), there was a significant positive association of the tumour grade with the CB₁IR intensity. The difference between the microsatellite stable and instable tumours for this association of CB₁IR was related to the CpG island methylation status of the cases. Cox proportional hazards regression analyses indicated a significant contribution of CB₁IR to disease-specific survival in the microsatellite stable tumours when adjusting for tumour stage. For the cases with stage II microsatellite stable tumours, there was a significant effect of both tumour centre and front CB₁IR upon disease specific survival. The 5 year probabilities of event-free survival were: 85±5 and 66±8%; tumour interior, 86±4% and 63±8% for the CB₁IR<2 and CB₁IR≥2 groups, respectively.

Conclusions/Significance: The level of CB₁ receptor expression in colorectal cancer is associated with the tumour grade in a manner dependent upon the degree of CpG hypermethylation. A high CB₁IR is indicative of a poorer prognosis in stage II microsatellite stable tumour patients.

Introduction

The G-protein coupled cannabinoid₁ (CB₁) receptors are most well known for their role in mediating the psychotropic effects sought after by recreational users of cannabis. However, CB₁ receptors and their endogenous ligands anandamide (arachidonylethanolamide) and 2-arachidonoylglycerol mediate a multitude of effects in the body including the regulation of pain, bone formation, energy homeostasis and gastrointestinal function [1–4]. In the human colon, CB₁ receptors are found in the crypt epithelium as well as in subepithelial inflammatory cells, in smooth muscle of blood vessels and in submucosal plexus [5,6], where they modulate, among other functions, the rate of intestinal transit and colonic propulsion [4].

In addition to the functions described above, the endocannabinoid system acts as a “damage limiting” system to mitigate the effects of pathological situations. This appears to be particularly true for the gastrointestinal endocannabinoid system. Thus, inflammation induced by agents such as 2,4-dinitrobenzene sulfonic acid, trinitrobenzene sulfonic acid, mustard oil or dextran sulfate sodium is more pronounced in CB₁ receptor knockout mice than in their wild type littermates, whilst treatment with a CB receptor agonist, or with compounds blocking the cellular removal and metabolism of endocannabinoids, alleviates the inflammation [7–11]. A CB₁ receptor polymorphism (1359 G/A) is associated with a reduced susceptibility to ulcerative colitis in man [12], although to our knowledge it is not yet known how this single nucleotide polymorphism affects cannabinoid signalling. Aberrant crypt foci

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in the colon, an early pathological change in the adenoma-carcinoma sequence in colorectal cancer development, are formed as a result of azoxymethane treatment in mice, and the treatment is associated with an increase in the levels of 2-arachidonoylgllycerol. Blockade of the metabolism of this endocannabinoid reduced the incidence of aberrant crypt foci, as did treatment with a CB receptor agonist [13] and, perhaps surprisingly, by treatment with a CB1 receptor inverse agonist [14].

Cannabinoids and endocannabinoids produce potentially useful effects upon cancer cell proliferation, motility and invasive behaviour (reviews, see [15,16]). In colorectal cancer cell lines, both CB1-dependent and -independent effects of endogenous and/or synthetic cannabinoids upon cell viability have been reported [17–20]. In a genetic model of colorectal cancer progression (Apc^Min/+ mice), animals lacking the CB1 receptor showed a greater number of small intestinal and colonic polyps than the corresponding CB1^+/+ mice [21]. Colorectal cancer patients who are either homo- or heterozygous for the 1359 G/A CB1 receptor polymorphism show a shorter survival time than the G/G wild-type patients [22]. Finally, reduced expression of CB1 receptor mRNA and protein have been reported in colorectal cancer [17,21], due at least in part to an increased rate of methylation of the promotor region of the receptor [21].

The above data are consistent with the suggestion that the endogenous cannabinoid system may be dysfunctional in colorectal cancer, and that such a dysfunction may affect disease severity and/or outcome. One way of investigating this possibility is to determine the level of CB1 receptor expression in a large cohort of well-characterised cases of colorectal cancer with long follow-up times. This has been undertaken in the present study. Given that colorectal cancer is highly heterogeneous, a particular focus has been made upon the relation of the CB1 immunoreactive intensities with key pathological/molecular components of the disease [23]: stage, tumour grade, microsatellite instability screening status, incidence of buds at the tumour front, and CpG island methylator phenotype (CIMP).

Methods

Ethics Statement

The research ethical committee at Umeå university hospital (Regional Ethical Review Board in Umeå, Sweden) approved the handling of tissue samples and patient data in the present study, including the procedure whereby patients verbally gave their informed consent. This consent was documented in each patient record, and this was considered by the ethical committee to be sufficient. In the database used for the analyses here, the tissue samples were given a case number and year, and the patient names were not indicated in the database.

Patients

The formalin-fixed, paraffin-embedded samples used in the present study were obtained from tissue collected during primary tumour surgical resection of colorectal cancer (CRUMS (Colorectal cancer in Umeå study)). The samples were collected with a consecutive intent at the Department of Surgery, Umeå University Hospital, Sweden, during the period 1995–2003 and where possible the patients were followed for up to 113 months [24]. In addition to the clinico-pathological data reported in [24], data on the microsatellite stability/instability screening status (immuno-chemical determination of the expression of four mismatch repair proteins) and CIMP have been collected and previously reported [25]. Incidence of buds at the tumour front were evaluated as in [26]. Exclusion criteria were insufficient or unavailable tumor tissue sample and insufficient clinical information. All in all, 487 cases were scored for either tumour centre or tumour front CB1 receptor immunoreactivity (see below). The clinical information in the database for these patients was as follows: median age 71 years (range 26–96, n = 487, of which 269 were males and 218 females); cancer location in right colon 31.1%, left colon 31.1%, rectum 37.8% (n = 482); disease stage I 15.5%, II 39.3%, III 21.0%, IV 24.2% (n = 476); tumour grade well/well-moderately differentiated 48.75%, moderate-poor/poorly differentiated 51.25% (n = 480); microsatellite stable 85.0%, microsatellite instable 15.0% (n = 473); CIMP status negative 50.4%, low 37.2%, high 12.4% (n = 484). Further, 82.4% (of 483 cases) did not receive preoperative radiotherapy (either 5x5 Gy or 25x2 Gy); 75.0% (of 476 cases) had radical surgery; and 13.7% (of 475 cases) received adjuvant chemotherapy.

Measurement of CB1 receptor immunoreactivity (CB1IR) in the tumour tissue

The paraffin-embedded tissue sections were deparaffinized and rehydrated before antigen retrieval in a pressure cooker (2100 retriever, Biocare Medical) in Diva Decloaker (Biocare Medical). Samples were subsequently placed in a Ventana semiautomated immunostaining machine (Ventana Medical Systems Inc., Tucson, AZ). The CB1 receptor antibody (AbCam cat. no. 23703, AbCam plc, Cambridge, UK, diluted 1:50) and the secondary components (iVIEW DAB Detection Kit, Ventana Medical Systems Inc.) were then added. The antibody, a rabbit polyclonal raised to an peptide corresponding to C terminal amino acids 461–472 of the human CB1 receptor and which cross-reacts with the human, mouse and rat CB1 receptor according to data from the manufacturers, has been shown previously by researchers in Umeå (including the corresponding author) to produce the appropriate pattern of staining in human cerebellum, but not to produce immunoreactivity in forebrains from CB1^−/− mice [27]. An ExPaSy Blast (http://expasy.org/tools/blast/) of the peptide sequence gave CB1 and its two splice variants as the only hits in man. In several other species, CB1 receptors were again identified, the only non-CB1 hits being from proteins termed “Uncharacterized protein [Gene: CNR1]” (from pig, dog and chicken) as well as “Putative uncharacterized protein [Gene: PANDA_015085] - Ailuroprocta melanoleuca (Giant panda)” and “Chromosome 14 SCAF15003, whole genome shotgun sequence fragment” in Tetraodon nigroviridis (Green pufferfish).

CB1IR was assessed by one investigator (SBG) who was blinded to the clinical status of the patients. The samples were graded on the basis of the dominant CB1 receptor immunoreactive intensity (CB1IR) in the tumour interiors and in the tumour invasive fronts and scored from 0 (absent) to 3 (intense) for the cells. When all samples had been scored, the investigator repeated the procedure (without access to the previous scores) and then compared the scores on the two runs. Cases where the scores were divergent were then assessed a third time, again without access to the previous scores. For the tumour front samples scored, for example, there were 53 occasions where the first and second scores disagreed, due mainly to erroneous scores from the initial stage of the first run. The final scores were then entered by another investigator (CJF) into the database for analysis.

Statistical evaluations

Kaplan-Meier survival analyses, Fisher’s exact test and χ2 tests were undertaken using the statistical package built into the GraphPad Prism 5 computer programme for the Macintosh (GraphPad Software Inc., San Diego, CA, USA). Cox proportional-hazards and binary logistic regression analyses were
Conducted using IBM SPSS statistics 19 software for the Macintosh (IBM Inc., Somers, NY, USA). For survival analyses, disease-specific events were defined as death with known disseminated or recurrent disease (‘dead’). Death from other causes was censored, as were the cases where the patient was still alive at the date of last follow-up. The duration of event-free survival is defined as the time from diagnosis until either the date of colorectal cancer death, death of other causes, or if no death occurred, until the date of last follow-up.

Results

CB₁IR immunohistochemistry

Initial studies were undertaken using the same batch of the antibody as in a previous study by researchers in Umeå using tissue microarrays from prostate cancer samples [27]. We found that at a dilution of 1:300, CB₁IR was found in the epithelial cells of the crypts, with scattered positivity in subepithelial inflammatory cells (Fig. S1A), a finding consistent with studies of CB₁IR in the normal colon [5,6]. An example of tumour tissue stained with this batch is shown in Fig. 1B. Due to limited amounts of antibody remaining, we used a later batch of the AbCam antibody (batch 761993) for the main study, and found that a lower dilution (1:50) was required for good immunostaining. An example of staining is seen in Fig. 1A, with a corresponding serial section without primary antibody shown in Fig. 1B.

The best specificity test for immunostaining is considered to be the use of knockout controls [28]. We found that appropriate immunochemical staining was seen in the brains of wild-type mice, whereas this was absent from brains of CB₁−/− mice (Fig. S2). Ashton [29] has recently argued that optical density histograms are useful in distinguishing between a change in the pattern of immunolabelling (as should be seen for a true loss of signalling in a knockout) compared to a reduction in labelling intensity (which would raise a question as to the validity of the antibody). Optical density histograms of our wild-type and CB₁−/− tissues are clearly different (best seen for the higher magnification slides, Fig. S2), consistent with good antibody specificity [29].

A common way of testing for antibody specificity is to investigate preadsorption of the antibody with the immunising peptide, although the usefulness of this measure has been questioned [30]. Nevertheless, a series of experiments using preincubation of the antibody with the immunising peptide (AbCam, Cat. No. 50542) were also undertaken. However, preincubation with the immunising peptide greatly increased the observed immunoreactivity rather than blocked it, and gave a rather random pattern of immunoreactive staining (data not shown). The immunising peptide (amino acid sequence MSVSTDTSANL) has two negatively charged amino acids (aspartate and glutamate), five polar amino acids (three serine, two threonine) and no positively charged amino acids. It is well known that negatively charged peptides bind to glass, and we conclude that the washing procedure in the Ventana technique, while being sufficient to provide good specificity per se (as seen in the knockout mice), is not sufficient to remove antibody-bound positive control peptide adhering non-specifically to glass and/or to cationic lipids.

Distribution of CB₁IR scores in the tumour centre and fronts

A total of 483 (tumour interior) and 486 (tumour front) cases were scored for CB₁IR intensity and entered into the database. Both plasma membrane and cytoplasmic CB₁IR was scored, so the values represent the total pool of CB₁ receptors. Nuclear staining of CB₁IR (found in 42 cases) was not scored. The frequency distributions of the CB₁IR for the tumour centre and fronts were similar, with a score of 0 being returned for 77 and 60 cases; a score of 1 for 196 and 185 cases; a score of 2 for 140 and 156 cases; and a score of 3 for 70 and 85 cases (numbers are tumour centre and fronts, respectively). There was no significant difference in the distribution pattern of the two sets of scores (p>0.1, χ² test). It was noted that the scores for the two regions were not always the same for a given case. Indeed, for the 482 cases scored for both tumour centre and front, the scores were the same in only 319 (66%) of the cases, being higher in the tumour front in 110 (23%) cases and higher in the tumour centre in 53 (11%) cases. In consequence, throughout this study, both tumour centre and tumour front scores have been analysed separately.

Non-malignant tissue was not scored, but in general its level of immunoreactivity appeared to be lower rather than higher than seen for the tumour tissue.

Association of CB₁IR with patient characteristics at surgery

In view of the frequency distribution of the CB₁IR, the analyses were conducted using a simple median split, i.e. CB₁IR<2 and ≥2. Using the entire data set to search for variables, a binary logistic regression with parameters gender, site (i.e. right colon, left colon, rectum), radiotherapy (prior to surgery), disease stage,

![Figure 1. CB₁ receptor immunoreactivity in tumour samples.](image-url)

Sections from the same case were used in the presence (Panel A) and absence (Panel B) of the primary antibody. Objective magnification is 10×.
tumour histological grade (i.e. differentiation), tumour type (i.e. mucinous or non-mucinous), microsatellite instability screening status [stable (MSS) or instable (MSI)], amount of lymphocytes at the tumour front and the frequency of tumour buds (small aggregates of tumour cells at the tumour invasion front, [31]) was conducted. Of these variables, only the tumour histological grade (p<0.005) and microsatellite instability screening status were significantly associated with the CB1IR (data not shown). These effects can simply be visualised by dividing the dataset into four groups on the basis of the two significant parameters (Fig. 2). The majority of the cases were classified as MSS, and the detailed CB1IR distributions for the MSS cases who did not receive radiotherapy prior to surgery are summarised in Table 1. In both the tumour centre and tumour fronts, there are more cases with a CB1IR≥2 for MSS cases with moderate-poor/poor tumour differentiation than with well/well-moderately differentiated tumours. In the MSI cases, this effect of the tumour histological grade is not seen, and the cases have a similar CB1IR distribution to the moderate-poor/poorly differentiated MSS cases (Fig. 2). Further analysis of the 62 cases with MSI scored for CB1IR who did not receive radiotherapy prior to biopsy revealed no significant association of the tumour centre or front CB1IR with either disease stage (here stages I and II were combined since only 4 cases with stage I were scored for tumour centre CB1IR), tumour grade, whether the tumours were mucinous or non-mucinous, incidence of lymphocytes at the tumour front or CIMP status (p>0.1, Fisher’s exact test or $\chi^2$ test, as appropriate, data not shown). For gender, the p values for the CB1IR distributions were 0.069 and 0.18 for tumour centre and front, respectively (Fisher’s exact test), and for the incidence of buds at the tumour front, the p values ($\chi^2$ test) were 0.086 and 0.073, respectively (data not shown).

One major difference between MSS and MSI is the greater incidence of a high degree of CpG island methylation in the latter [32]. In our data set, of the 467 cases scored for microsatellite instability screening status, CIMP status and tumour centre CB1IR, the CIMP distributions were: MSS (n = 396), negative 225 (57%), low 153 (39%) and high 18 (4.5%); MSI (n = 71), negative 15 (21%), low 17 (24%) and high 39 (55%) (p<0.0001, $\chi^2$ test). When the CIMP status of the samples was added into the binary logistic regression of the whole dataset with the parameters described above, the significant effect of the tumour histological grade was retained (p<0.005) for both tumour centre and front CB1IR, but the effect of the microsatellite instability screening status was lost for the tumour centres (p>0.1) but not for the tumour fronts (p<0.05) (data not shown). In order to investigate this further, the data for the samples was divided into the three CIMP groups: negative, low and high regardless of microsatellite instability screening status or whether the patients had received radiotherapy prior to surgery. There was a clear influence of CIMP status on the results, where the effect of the tumour histological grade was seen in the cases with a CIMP-negative score but not in the cases with a CIMP-high score, the CIMP-low cases being somewhere in the middle (Fig. 3). This analysis did not take into account the microsatellite instability screening status of the cases in order to achieve sufficient group sizes. However, a similar pattern is seen when the MSS and MSI cases are analysed separately, although as a caveat it should be pointed out that some of the group sizes are very small (Fig. S3).

**Association of CB1IR with disease outcome**

The cases in the database had been followed for up to 113 months [24] allowing the influence of the CB1IR score at diagnosis upon disease outcome to be determined. Using the entire dataset (i.e. even those cases where CB1IR was not scored, a univariate Cox proportional hazards regression analysis on the entire dataset indicated that the administration of radiotherapy prior to surgery was, unsurprisingly, associated with disease outcome (Exp(B) 0.60 [95% CI 0.40–0.91] p<0.05) where the number of cases not receiving/receiving radiotherapy was 441/102. The Cox proportional hazards regression analysis is a test used to determine the contribution of the parameter(s) under investigation upon disease-specific survival without making assumptions as to the nature of

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**Figure 2. Influence of the tumour histological grade and MSI screening status upon CB1IR in colorectal cancer.** Panel A, tumour centre; Panel B, tumour front. The data are grouped according to tumour histological grade (w-m, well/well-moderately differentiated; m-p, moderate-poor/poorly differentiated) and microsatellite stability (MSS, stable; MSI, instable). The $\chi^2$ and hence p values are for the data grouped as a 4×2 matrix (where the 2 is the CB1IR). n refers to the total number of cases for each bar.

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the survival curve. The measure Exp(B), sometimes called the hazards ratio, indicates the change in risk as the parameter under investigation is changed from the default parameter (here no radiotherapy) to the test parameter (here radiotherapy). In this case, the radiotherapy reduced the risk of death due to the disease since the Exp(B) value was significantly lower than unity. In order to remove the influence of this parameter, the CB1IR data was analysed only for the cases that did not receive radiotherapy. In addition, the MSS and MSI cases were analysed separately.

For the MSS cases, a univariate Cox proportional hazards regression analysis failed to show a significant effect of either tumour interior or front CB1IR upon disease-specific survival. However, when a bivariate analysis was undertaken with disease stage as the second parameter, a highly significant contribution of CB1IR was seen (Table 2). This was confirmed in Kaplan-Meier survival plots: for the entire data set, there was no significant contribution of the tumour interior CB1IR (Fig. 4A), whereas in tumour stage II (where the tumours have infiltrated the muscularis propria of the colon or rectum, but have not given rise to lymph node metastasis), and in tumour stage IV (where the tumours have spread to other organs), cases with a CB1IR > 2 have a poorer disease-specific survival than cases with a CB1IR ≤ 2 (Figs. 4B and D; definitions of disease staging given in [33]). No difference was seen for tumour stage III, although it should be borne in mind that this is a highly heterogenous group, that the number of cases was smaller than for disease stages II and IV and that subgroup

| Parameter       | Tumour centre | Tumour front |
|-----------------|---------------|--------------|
|                 | n  | CB1IR<2 | CB1IR≥2 | p  | n  | CB1IR<2 | CB1IR≥2 | p  |
| Age (y)         | 318 | 72 [26–96] | 74 [35–89] | 0.14* | 321 | 72 [26–93] | 72 [35–96] | 0.49* |
| Gender          |     |         |         |      |     |         |         |      |
| Males           | 175 | 107 [61] | 68 [39] | 0.91b | 178 | 104 [58] | 74 [42] | 0.43b |
| Females         | 143 | 86 [60] | 57 [40] |      | 143 | 77 [54] | 66 [46] |      |
| Site            |     |         |         |      |     |         |         |      |
| Right colon     | 92  | 57 [62] | 35 [38] | 0.40c | 95  | 48 [51] | 47 [49] | 0.22c |
| Left colon      | 136 | 77 [57] | 59 [43] |      | 135 | 74 [55] | 61 [45] |      |
| Rectum          | 87  | 57 [66] | 30 [34] |      | 87  | 55 [63] | 32 [37] |      |
| Disease stage   |     |         |         |      |     |         |         |      |
| I               | 40  | 27 [68]% | 13 [33]% | 0.31c | 40  | 23 [58]% | 17 [43]% | 0.57c |
| II              | 123 | 74 [60] | 49 [40] |      | 125 | 74 [59] | 51 [41] |      |
| III             | 59  | 30 [51] | 29 [49] |      | 60  | 29 [48] | 31 [52] |      |
| IV              | 89  | 57 [64] | 32 [36] |      | 89  | 49 [55] | 40 [45] |      |
| Tumour grade    |     |         |         |      |     |         |         |      |
| w-m             | 170 | 116 [68] | 54 [32] | 0.0052b | 171 | 113 [66] | 58 [34] | 0.0001b |
| m-p             | 143 | 75 [52] | 68 [48] |      | 145 | 64 [44] | 81 [56] |      |
| Tumour type     |     |         |         |      |     |         |         |      |
| Mucinous        | 41  | 26 [63] | 15 [37] | 0.86b | 42  | 20 [48] | 22 [52] | 0.24b |
| Non-mucinous    | 273 | 165 [60] | 108 [40] |      | 275 | 159 [58] | 116 [42] |      |
| Lymphocytes (at TF) |     |         |         |      |     |         |         |      |
| Low no.         | 168 | 99 [59] | 69 [41] | 0.64b | 171 | 88 [51] | 83 [49] | 0.069b |
| High no.        | 145 | 90 [62] | 55 [38] |      | 145 | 90 [62] | 55 [38] |      |
| buds (at TF)    |     |         |         |      |     |         |         |      |
| None            | 20  | 11 [55] | 9 [45] | 0.66c | 20  | 8 [40] | 12 [60] | 0.054c |
| 1–9             | 132 | 83 [63] | 49 [37] |      | 133 | 85 [64] | 48 [36] |      |
| 10–19           | 79  | 45 [57] | 34 [43] |      | 79  | 38 [48] | 41 [52] |      |
| ≥20             | 75  | 49 [65] | 26 [35] |      | 78  | 46 [59] | 32 [41] |      |
| CIMP status     |     |         |         |      |     |         |         |      |
| Negative        | 173 | 106 [61] | 67 [39] | 0.83c | 174 | 99 [57] | 75 [43] | 0.85c |
| Low             | 128 | 78 [61] | 50 [39] |      | 129 | 74 [57] | 55 [43] |      |
| High            | 15  | 8 [53] | 7 [47] |      | 16  | 8 [50] | 8 [50] |      |

Abbreviation: TF, tumour front; CIMP, CpG island methylator phenotype. w-m, well/well-moderately differentiated, m-p, , moderate-poor/poorly differentiated.

aData for age is given as medians with range, and the p value was from a Mann-Whitney U-test.
bp values determined by Fisher’s exact test.
cValues determined by x2 test.
dThe rounding up of the % (e.g. 67.5% → 68%) gives the sum total of 101% for the tumour centre and front data.

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| CB1 Receptor Immunoreactivity in Colorectal Cancer

Table 1. CB1IR in tumour interiors for microsatellite stable (MSS) cancers: correlation with patient characteristics for patients not receiving radiotherapy prior to surgery.
The present study was motivated by data from both cultured cells and patient samples suggesting that a dysfunctional endocannabinoid signalling system is involved either in the pathogenesis and/or as a consequence of colorectal cancer [13,14,17,21,22]. At the outset it is worth commenting upon the fact that both cell surface and cytoplasmic CB1 receptors were scored. CB1 receptors are often regarded as cell surface receptors, but it is well established in many cells and tissues that they have been found intracellularly [34-37], as would be expected for receptors that internalise following sustained agonist stimulation [38,39]. It has been suggested that these intracellular receptors are active and couple to extracellular signal-regulated kinase [35], although other authors have not seen intracellular co-localisation of CB1 receptors with G5 subunits [39]. Naturally-occurring ligands for CB1 receptors are highly lipophilic, and thus the plasma membrane is not a barrier to their cellular penetration. Extracellular signal-regulated kinase is an important signalling molecule, and has been implicated in antiproliferative effects of cannabinoids in a number of different cancer cell lines [40], so it is not unreasonable (and technically considerably less difficult) to score the combined intensity from plasma membrane and intracellular CB1 receptors. There are three main results in from the study, and these are discussed in turn.

**Discussion**

The present study was motivated by data from both cultured cells and patient samples suggesting that a dysfunctional endocannabinoid signalling system is involved either in the pathogenesis and/or as a consequence of colorectal cancer [13,14,17,21,22]. At the outset it is worth commenting upon the fact that both cell surface and cytoplasmic CB1 receptors were scored. CB1 receptors are often regarded as cell surface receptors, but it is well established in many cells and tissues that they have been found intracellularly [34-37], as would be expected for receptors that internalise following sustained agonist stimulation [38,39]. It has been suggested that these intracellular receptors are active and couple to extracellular signal-regulated kinase [35], although other authors have not seen intracellular co-localisation of CB1 receptors with G5 subunits [39]. Naturally-occurring ligands for CB1 receptors are highly lipophilic, and thus the plasma membrane is not a barrier to their cellular penetration. Extracellular signal-regulated kinase is an important signalling molecule, and has been implicated in antiproliferative effects of cannabinoids in a number of different cancer cell lines [40], so it is not unreasonable (and technically considerably less difficult) to score the combined intensity from plasma membrane and intracellular CB1 receptors. There are three main results in from the study, and these are discussed in turn.

The fact that in the MSS patients the CB1IR is associated with the tumour histological grade at surgery may of course mean that the association described above is simply a reflection of the influence of the tumour histological grade upon disease outcome. This possibility was investigated using multivariate Cox proportional hazards regression analyses (Table 2), where it was found that the tumour histological grade provided additive prognostic information to that seen with the tumour centre CB1IR when the disease stage was also included as a parameter. In contrast, the tumour front CB1IR parameter lost significance. However, when the incidence of bads at the tumour front was also included (in itself a prognostic factor), the influence of the tumour histological grade upon disease outcome was reduced, whereas the influence of both tumour center and front CB1IR was significant (Table 2). These data would suggest that the prognostic significance of the tumour centre and possibly also tumour front CB1IR is not secondary to its association with the tumour histological grade. Further analysis indicated that the prognostic significance of CB1IR remained when the CIMP status, gender and tumour region were included in the multivariate analysis (tumour centre, Exp(B) 1.77 [95% CL 1.16–2.72, p<0.01; tumour front Exp(B) 1.67 [95% CL 1.11–2.50] p<0.05).

For the MSI cases, no conclusions could be drawn as to whether CB1IR impacted upon disease-specific survival in stage II cases, simply because of the 32 cases that fell into this category (after exclusion of cases receiving radiotherapy prior to surgery), only one died of the cancer during the follow-up period.

**Figure 3. Patients with CIMP-high tumours have CB1IR levels that are not dependent upon the tumour grade.** Panel A, tumour centre; Panel B, tumour front. The data are grouped according to tumour histological grade (w-m, well/well-moderately differentiated; m-p, moderate-poor/poorly differentiated) and the CIMP status. Of the 473 cases scored for tumour centre CB1IR, 389 were classified as MSS, 71 as MSI and 13 not classified in the data base. The corresponding numbers for the tumour front CB1IR were 392, 71 and 13, respectively. The p values are for Fisher’s exact test. The total (i.e. CB1IR<2 and ≥2) number of cases is shown enclosed within each bar. doi:10.1371/journal.pone.0023003.g003

analyses suffer from low power. There were only three patients diagnosed with stage I colorectal cancer (where the tumour has only infiltrated the submucosal layer of the colon or rectum and has not spread to the lymph nodes or other organs) who died as a result of their cancer during the follow-up period, precluding analysis of the influence of CB1IR upon disease-specific outcome in this patient group. In the case of the tumour front CB1IR, the significant effect upon tumour outcome in the stage II patients was also seen, but not in the stage IV patients (Fig. 4 and data not shown, respectively). In the stage II cases, the 5 year probabilities of event-free survival were: 85±5 and 66±8%; tumour interior, 86±4% and 63±8% for the CB1IR<2 and CB1IR≥2 groups, respectively. It can be noted that some of the stage II cases died within one month of surgery. However, when these cases were excluded, the significant contribution of CB1IR to the disease-specific outcome was retained (data not shown). Upon further subdivision of the stage II cases according to cancer site, a significant contribution of both tumour centre and front CB1IR upon disease-specific survival was seen for the rectal cancers, but not for the colon cancers, although the direction (i.e. poorer survival for a high CB1IR) was the same. It is important to note, however, that interpretation of results with these subgroups are limited by a low power and are not supported by the Cox analyses (see below).
1. Tumour centres and invasive fronts have different CB1IR intensities in one third of cases investigated

For both tumour centres and invasive fronts, a gamut of CB1IR scores from absent (0) to pronounced (3) were seen. Although there was no significant difference between the CB1IR distribution patterns for the two regions, one third of the cases had a score for the tumour invasive front that was different from that for the corresponding tumour centre CB1IR. One possible explanation for this difference is that the tumour front is an area of intense immunological and inflammatory activity [41], and it can be hypothesised that constituents of the tumour front microenvironment affect the transcription of CB1 receptors in this region. One

| Table 2. Cox proportional-hazards regression analyses for microsatellite stable (MSS) cancers; influence of disease stage, tumour grade and number of tumour front buds. |
|-------------------|-------------------|-------------------|
| Variable          | Tumour centre     | Tumour front      |
|                   | Cat. value        | n    | Exp(B) [95%CL] | n    | Exp(B) [95%CL] |
| CB1IR             | < 2 (1)           | 182  | 1              | 170  | 1              |
|                   | ≥ 2 (2)           | 121  | 1.21 [0.84–1.75]** | 136  | 1.36 [0.94–1.95]** |
| Disease Stage     | I (1)             | 40   | 40             | 40   | 40             |
|                   | II (2)            | 121  | 2.50 [0.75–8.35]** | 123  | 2.67 [0.80–8.86]** |
|                   | III (3)           | 58   | 4.08 [1.19–14.0]** | 59   | 4.14 [1.20–14.2]** |
|                   | IV (4)            | 84   | 41.0 [12.7–132]***** | 84   | 38.0 [11.8–122]***** |

Univariate analyses

Bivariate analyses

Multivariate analyses

Abbreviations: TF, tumour front; w-m, well/well-moderately differentiated; m-p, moderate-poor/poorly differentiated; Cat. value, categorical value. Exp(B) refers to the increase in the odds as a result of an increase in the “unit” (shown in brackets in the categorical value column). Significance levels:

***p < 0.001,
**p < 0.01,
*p < 0.05,
*p < 0.1.

In a separate univariate analysis, the Exp(B) value (with 95% confidence limits) for the differentiation state parameter was 2.08 [1.44–3.00], p < 0.001. For a bivariate analysis with disease stage and differentiation state, the Exp (B) value (with 95% confidence limits) for the differentiation state parameter was 1.60 [1.10–2.33], p < 0.05.

The level of significance was retained when no. of TF buds was added as a third parameter. Finally, in multivariate analyses with disease stage, no. of TF buds and CB1IR score, the Exp(B) value (with 95% confidence limits) for tumour centre and tumour front CB1IR scores were 1.97 [1.31–2.96], p < 0.01 and 1.81 [1.23–2.68], p < 0.01, respectively.

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potential candidate in this respect is the cytokine interleukin-4 (IL-4), given that it can increase CB1 receptor expression [42], and that both IL-4 and IL-4 receptors are found in colon tumours [43,44]. It would be clearly of interest to determine whether other constituents of the tumour microenvironment affect CB1 receptor transcription.

2. The tumour histological grade is associated with CB1IR in a manner modulated by the CIMP status

For MSS cases, well/well-moderately differentiated tumours distribute with the ratio of CB1IR<2:≥2 among the cases of approximately 2:1, whilst the ratio is close to 1:1 for the cases with moderate-poor/poorly differentiated tumours at surgery. In the MSI cases, no such difference is seen and the cases have a ratio near 1:1. MSI cases are characterised by a high incidence of mutated DNA microsatellite markers as a result of a loss of DNA mismatch repair, and differ from MSS cases not only in terms of tumour characteristics and gene expression profiles [45] but also in the survival rates and responses to chemotherapy [46]. It is thus perhaps not surprising that the MSS and MSI cases have different CB1IR distributions. However, a major difference between MSS and MSI cases are the relative incidences of CIMP-negative, low and high [32], and our analysis suggests that the CIMP status rather than the microsatellite stability is a prime determinant of the association of tumour grade with CB1IR. It is notable that for all cases with a negative CIMP status and moderate-poorly differentiated tumours, the relative proportion of cases with CB1IR≥2 is similar to that seen for well/well-moderately and for moderate-poor/poorly differentiated CIMP-high tumours. This would suggest that the shift in CB1IR distribution seen with histological grade is brought about along the same pathway as the shift seen with CIMP-high, so that the effects are not additive.

With respect to the effects of DNA hypermethylation upon CB1 receptor expression, Wang et al. [21] investigated in a series of 13 cases the methylation status of 39 cytosine and guanine-rich DNA segments (“CpG islands”) in the region (−212 to +140) of the start...
site for transcription of the 
*Cur1* gene (the gene responsible for the
CB1 receptor). These authors found hypermethylation of these
sites in the colorectal tumours, ranging from cases with a single
action at position +108 to cases with hypermethylation in ≥15
sites. They reported that the hypermethylation resulted in *Cur1*
gen silencing [21]. Although their study shows an effect in the
opposite direction to the apparent effect of a CIMP-high score
seen here, it is important to stress that the determination of CIMP
in our study was based on a validated eight gene screening panel
[25] and is thus an indication that the tumours have a high
frequency of hypermethylated genes in general, whereas Wang et
al [21] focused on methylation of sites directly relevant to the
*Cur1* gene. It is possible that one or several genes that are commonly
inactivated by hypermethylation in CIMP-high tumours have
downstream effects upon the transcription and/or turnover of the
CB1 receptor, and this produces the results seen here, or
alternatively that the pattern of *Cur1* hypermethylation in
colorectal cancer is rather different from that picked up in the
CIMP screen. In the latter situation, a case with a low level of CB1
promotor methylation but a high CIMP score (or vice versa)
would be classified differently in the two studies, so it is not surprising that
the results are divergent. In future studies, it would clearly be of
interest to investigate *Cur1* hypermethylation in our tissue material,
to be able to distinguish between these alternatives.

3. A high CB1IR is associated with a poorer disease-
specific survival in patients with stage II MSS colorectal
cancer

From the introduction, it might have been expected that a high
CB1 tumour receptor expression would be beneficial to the
patients, whereas the opposite was found to be the case, at least for
the patients with stage II MSS tumours at surgery. To our
knowledge, only three studies have been undertaken to investigate
the prognostic value of CB1IR in solid tumours. In hepatocellular
carcinoma, the 35 cases with an undetectable or faint CB1IR
showed a significantly poorer disease-free survival than the 29
cases with a moderate or intense CB1IR [47]. Interestingly, the
distribution of CB1IR was also associated with histological grade,
with 20/34 of the cases with well/well-moderately differentiated
tumours showing a high CB1IR while only 9/30 cases with
moderate-poor/poorly differentiated tumours showed a high
CB1IR [47]. The other two studies, one in pancreas cancer and
one in prostate cancer, are consistent with the present study. In
the pancreatic cancer study, two cohorts were used. In the first cohort
(n=37), a composite scale of immunoreactive intensity × distribution
was used, and cases with a high score were found to have a
significantly shorter median survival than those with a low score
[48]. The same result was seen in a second cohort (n=53)
measuring CB1 receptor mRNA expression with quantitative RT-
PCR. In the prostate cancer study (conducted in Umeå using
the same antibody as in the present study, albeit a different batch), a
composite score was again used, and the disease-specific survival
was significantly poorer for the 192 cases with a CB1IR equal to or
above the median (15 year probability of event-free survival
50±5%) than for the 77 cases with a CB1IR below the median (15
year probability of event-free survival 78±7%) [26].

It is of course naive to assume that the influence of the CB1IR
score is going to be the same regardless of the cancer in question,
but the present study would suggest that hepatocellular carcinoma,
rather than colorectal cancer, is the “odd cancer out”. The
question nevertheless remains as to why a high, rather than a low,
CB1 expression should be associated with a poorer disease-specific
survival. One possible explanation has been furnished by a recent
study using cultured astrocytoma cells transfected with CB1
receptors [49]. In that study, the authors selected clones with
different CB1 receptor expression levels and found that at a low
receptor expression, the receptors coupled primarily to extracel-
ulusar signal-regulated kinases, and that activation of the CB1
receptors led to apoptosis. In contrast, at a high level of CB1
receptor expression, activation of the receptors led additionally to
the activation of the Akt survival pathway, and cannabinoids only
produced apoptosis when this pathway was inhibited [49]. It is of course a long way from studies in transfected cells to the situation
in solid tumours, but the postulation that a high CB1 receptor
expression results in the switch from a pro-apoptotic to a
predominantly pro-survival pathway would mean that the local
endocannabinoid tone no longer acts to limit the damaging
influence of the tumour but rather to exacerbate it and thereby
result in a poorer prognosis for the patient. The hepatocellular
carcinoma data [47] can be incorporated into this admittedly
speculative hypothesis by suggesting that the cancers defined as
high CB1IR do not have a sufficiently high level of expression to
trigger the switch in these cells.

A final note concerns the potential of CB1IR as a prognostic
marker to aid treatment decisions in cancer. In prostate cancer,
CB1IR looks to be a very promising marker that provides additive
prognostic information to that supplied by other variables such as
the Gleason score and the tumour stage [27,50]. For colorectal
cancer, the situation is less promising, since prognostic significance
was not across the board, but unmasked in the MSS cases when
the disease stage was also taken into consideration. Nonetheless,
given that patients with stage II colorectal cancers are a patient
group where treatment decisions are difficult and better prognostic
markers are needed [51], the present data warrant further
investigation into the potential usefulness of CB1IR as a prognostic
marker to aid such treatment decisions in stage II MSS colorectal
cancer.

Supporting Information

**Figure S1 CB1 receptor immunoreactivity in non-malignant and adenocarcinoma samples.** Panel A, non-
malignant tissue; Panel B adenocarcinoma tissue, both stained
using the antibody batch used in [27]. Objective magnification is
10×.

**Figure S2 CB1 receptor immunoreactivity in forebrain samples from wild-type and CB1 receptor knockout
mice.** Panels A and B show the immunoreactivity from wild-type
and CB1 receptor knockout mice, respectively. Objective magnification is 1.25×. The tiff image from the selected areas was
imported into Adobe Photoshop (version CS4 for the Macintosh)
and the colour histograms were captured. Panels C (wild-type) and
D (CB1 receptor knockout) show immunoreactivity from different
forebrain tissue slides to those in Panels A and B, at a higher
objective magnification (20×). The colour histograms are for the
whole images. The paraffin embedded, formalin-fixed mouse
tissue was kindly provided by Drs. Beat Lutz and Giacomo
Mancini, Department of Physiological Chemistry, Johannes
Gutenberg-University Mainz, Germany.

**Figure S3 Division of CB1IR scores according to tumour
grade, CIMP status and microsatellite stability screening
status.** Panel A, tumour centre; Panel B, tumour front. The
data are grouped according to tumour grade (w-m, well/well-
moderately differentiated; m-p, moderate-poor/poorly differen-
tiated) and microsatellite stability (MSS, stable; MSL, instable) and

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the CIMP status. P values were determined using Fisher's exact test. The total (i.e. CB1/IR<2 and ≥2) number of cases is shown enclosed within each bar.

(TIF)

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**Author Contributions**

Conceived and designed the experiments: RP SOPJ CJF. Performed the experiments: SBG RP. Analyzed the data: CJF RP. Contributed reagents/materials/analysis tools: MLH AMD SE AO. Wrote the paper: SBG RP CJF.

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