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The calcium-sensing receptor: A promising target for prevention of colorectal cancer☆

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ABSTRACT

The inverse correlation between dietary calcium intake and the risk of colorectal cancer (CRC) is well known, but poorly understood. Expression of the calcium-sensing receptor (CaSR), a calcium-binding G protein-coupled receptor is downregulated in CRC leading us to hypothesize that the CaSR has tumor suppressive roles in the colon. The aim of this study was to understand whether restoration of CaSR expression could reduce the malignant phenotype in CRC.

In human colorectal tumors, expression of the CaSR negatively correlated with proliferation markers whereas loss of CaSR correlated with poor tumor differentiation and reduced apoptotic potential. *In vivo*, dearth of CaSR significantly increased expression of proliferation markers and decreased levels of differentiation and apoptotic markers in the colons of *CaSR/PTH* double knock-out mice confirming the tumor suppressive functions of CaSR. In *vitro* CRC cells stably overexpressing wild-type CaSR showed significant reduction in proliferation, as well as increased differentiation and apoptotic potential. The positive allosteric modulator of CaSR, NPS R-568 further enhanced these effects, whereas treatment with the negative allosteric modulator, NPS 2143 inhibited these functions. Interestingly, the dominant-negative mutant (R185Q) was able to abrogate these effects.

Our results demonstrate a critical tumor suppressive role of CaSR in the colon. Restoration of CaSR expression and function is linked to regulation of the balance between proliferation, differentiation, and apoptosis and provides a rationale for novel strategies in CRC therapy.

1. Introduction

Garland et al. demonstrated an inverse correlation between dietary calcium (Ca^{2+}) intake and the risk of colorectal cancer (CRC), identifying nutritional Ca^{2+} as a promising chemopreventive agent [1]. Since then several advances have been made with focus on studies trying to establish risk factors to facilitate suitable intervention in CRC [1–3]. In the colon, Ca^{2+} exerts its functions through various mechanisms, including binding toxic bile acids and ionized fatty acids, to form insoluble soaps or by regulating cellular proliferation, inducing differentiation and/or stimulating apoptosis [4–12]. However these anti-tumorigenic effects of Ca^{2+} fail during tumor progression. Although it is clear that Ca^{2+} exerts chemopreventive features in the colon, the molecular mechanisms by which extracellular Ca^{2+} modulates cell fate are not fully understood. Finding markers that would allow us to distinguish between an individual's responsiveness to calcium, or a target that could be modulated to restore responsiveness is of utmost importance.

The calcium-sensing receptor (CaSR) has been identified as a key molecule in regulating systemic calcium homeostasis in the parathyroid [13]. The *CaSR* gene encodes a calcium-binding G protein-coupled receptor (GPCR), with an extracellular N-terminal domain (containing the calcium binding sites), joined to the C-terminal domain via a seven transmembrane region (essential for its signaling function). Most orthosteric ligands of the CaSR bind to the large N-terminal

Abbreviations: Ca^{2+}, calcium; CRC, colorectal cancer; CaSR, calcium-sensing receptor; PTH, parathyroid hormone; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; Zeo, Zeocin; RPLPO, large ribosomal protein; β2M, beta-2-microglobulin; Eef1B2, eukaryotic translation elongation factor 1 beta 2; ANOVA, analysis of variance; CDC, cell division cycle; CDT1, chromatin licensing and DNA replication factor 1; MCM, mini chromosomal maintenance complex; CDX2, caudal type homeobox 2; SI, sucrase isomaltase; BAX, Bcl-2 associated X protein; SCC, Spearman correlation coefficient; EMP, empty; WT, wild type; DN, dominant negative

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domain of the receptor [14]. Several synthetic modulators of the CaSR have been developed in order to modulate CaSR function. Calcimimetic agents like NPS R-568 are positive allosteric modulators of the CaSR, which potentiate the effects of the CaSR by interacting with the 7-transmembrane region of the receptor and inducing conformational changes. Calcilytic agents (e.g. NPS 2143) that are negative allosteric modulators of the receptor act in a similar manner, desensitizing the receptor, and reducing its affinity to its ligands [14,15].

There is strong evidence for the involvement of the CaSR in various functions determining cellular fate [4,16,17]. These functions extend beyond the control of calcium homeostasis; the primary function of the CaSR. It has been suggested that the CaSR is either a tumor suppressor (e.g. in colon and parathyroid) or an oncogene (e.g. in breast and prostate) depending on the site of disease [18]. Expression of colonic CaSR is significantly downregulated during colorectal tumorigenesis [19–21] at least in part, by aberrant DNA methylation and histone deacetylation [19,22]. CRC cells that lack the CaSR have a highly malignant phenotype [23]. Taken together, the epidemiological observation of the inverse relationship between Ca^{2+} intake and risk of CRC, the observation that CaSR mRNA and protein expression is reduced in human colon tumors, and the observations that CRC cells lacking the CaSR have a malignant phenotype lead to the hypothesis that the CaSR is a tumor suppressor in the colon. However, there are only limited or no data in support of a causal relationship between CaSR expression and de-differentiation and carcinogenesis.

In this study we present evidence that the CaSR is a tumor suppressor in the colon using three distinct approaches: *ex vivo*, *in vivo* and *in vitro*. These models have allowed us to investigate the role of the receptor in the colon and to understand whether restoration of CaSR function can prevent or attenuate the malignant phenotype in CRC.

2. Materials and methods

2.1. Human patient samples

Fresh frozen tumor tissue and adjacent non-tumor tissue from 54 CRC patients were obtained after written consent. Samples were collected from the General Hospital of Vienna and Rudolfstiftung Hospital in Vienna and snap frozen in liquid nitrogen. A pathologist graded and classified the samples according to the TNM system. Approval by the local ethics committees was obtained prior to the start of the study. Clinicopathological characteristics of the patient cohort are shown in Table 1.

Table 1
Clinicopathological characteristics of the patient cohort.

Patient characteristics	
Number	54
Gender	
Male	28
Female	26
Age (Mean ± SD)	68.7 ± 12.2
Tumor grading	
Grade 1	1
Grade 2	51
Grade 3	1
Unknown	1
Tumor staging	
Stage 1	11
Stage 2	17
Stage 3	25
Stage 4	1
Lymph node infiltration	
0	27
1	13
2	11
3	1
Unknown	2
Site of primary tumor	
Cecum/ascending/transverse	22
Descending/sigmoid	19
Rectum	13

2.2. Animals

Mice heterozygous for $CaSR^{\Delta Exon5}$ and *PTH* were bred to generate $CaSR^{+/+}/PTH^{-/-}$ and $CaSR^{-/-}/PTH^{-/-}$ mice as previously described [24]. Ethics approval was obtained from the Institutional Animal Care and Use Committee at Harvard Medical School. Mice were fed standard mouse chow (Harlan Teklad-TD99224, Harlan-Teklad, USA). Age- and sex-matched animals (n = 9/genotype) were sacrificed, the colons were washed in ice cold PBS and stored in RNAlater (Life Technologies, Austria) until RNA extraction was carried out. For the analysis, 1–2 cm of colonic tissue 0.5 cm distal from cecum was used.

2.3. RNA isolation, reverse transcription and quantitative-reverse transcription PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA integrity was checked by agarose gel electrophoresis, and total RNA was reverse transcribed as previously described [25]. qRT-PCR was performed using POWER SYBR Green Mastermix (Life Technologies) on a StepOne Plus qRT-PCR machine (Life Technologies). Where possible, primers were designed to bridge an exon–exon junction to prevent genomic DNA from being amplified. The $\Delta\Delta C_t$ method was used to calculate fold changes in gene expression, relative to housekeeping genes (human beta-actin (hβ-ACTIN) for human patient samples; hβ-ACTIN, human large ribosomal protein (hRPLPO) and human beta-2-microglobulin (hβ2M) for human cell lines; mβ-Actin and mouse eukaryotic translation elongation factor 1 beta 2 (mEef1b2) for mouse colon samples) and normalized to a commercially available total RNA calibrator (Clontech, USA). Primer sequences are described in Supplementary Table S1. Primer sequences for hCDC6, hMCM2, hβ-ACTIN, hRPLPO and hβ2M have been previously described [19,25].

2.4. Cell culture

The human CRC cell lines Caco2-15 and HT29 were used in this study. HT29 cells were obtained from American Type Culture Collection (ATCC, USA). The Caco2-15 cells are a sub-clone of Caco2 cells [26] and were kindly provided by Prof. A Quaroni (Cornell University, USA). Cells were regularly maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) as previously described [19]. Ca^{2+} concentration in the medium was 1.8 mM, unless otherwise stated. Cells were routinely tested for mycoplasma contamination using the VenorGem Mycoplasma Detection Kit (Minerva Biolabs, Germany) and were periodically authenticated by STR DNA profiling (DNA Diagnostic Center, UK).

2.5. Cloning of the human CaSR cDNA

pcDNA3.1 expression vector encoding a full length wild-type CaSR (CaSR-WT) was kindly provided by Dr. AL Magno and Prof. BK Ward (University of Western Australia, Nedlands). hCaSR cDNA was subcloned into the HindIII and XbaI restriction enzyme sites of the pcDNA™3.1/Zeo$^{(+)}$ expression vector (Life Technologies). The hCaSR sequence was FLAG-tagged in its C-terminal region using a reverse primer designed to contain, in the 5′ to 3′ direction, the XbaI recognition sequence, the wild-type stop codon sequence, the sequence encoding the FLAG peptide (GACTACAAGGACGACGATGACAAG), and the hCaSR sequence adjacent to the wild-type stop codon. Another set of primers was then used to generate the R185Q dominant negative mutant of