The mRNA of L-Type Calcium Channel Elevated in Colon Cancer

Protein Distribution in Normal and Cancerous Colon

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Previous reports indicate that the mRNA for the cardiac isoform of the voltage-gated L-type calcium channel (α_{1C}) is elevated in colon cancer. The aim of these experiments was to verify that the mRNA for α_{1C} was significantly increased in tumors of two separate populations of patients when compared to normal adjacent mucosa. The second aim was to measure the distribution of α_{1C} using immunocytochemistry in normal human colon and in colon cancer and to determine what might regulate the channel expression. Biopsies were taken from patients with various stages of colon cancer and nearby normal mucosa were used as control. RNA was prepared and mRNA level measured by semiquantitative reverse transcriptase-polymerase chain reaction. The mRNA of the calcium channel was compared with other markers including β -actin. The mRNA for α_{1C} was increased significantly in colon cancers compared to nearby adjacent mucosa. Using confocal microscopy α_{1C} was localized mainly at the apical membrane in the surface epithelium of normal human colon with less distribution on the lateral and basal membranes. The channel was localized on the lateral and basal membranes in crypt cells. Calcium channel localization appeared to be nearer nuclei in colon cancer samples, in part because of the smaller size of the cells. Likewise, cultured Caco-2 and T84 cells showed a membrane distribution. Western blotting indicated that α_{1C} protein was increased in nonconfluent cultures of colonic carcinoma cells compared to confluent cells and immunocytochemistry confirms that there is more calcium channel protein in cells that are nonconfluent. We conclude that the increase in mRNA of α_1 subunit of the cardiac isoform of the L-type calcium channel may be a useful marker of

colon cancer compared to other markers because the increase is large and this increase can be documented on small samples using a simple semiquantitative reverse transcriptase-polymerase chain reaction. We found that α_{1C} protein is increased when colonic cells are nonconfluent or dividing which may account for the increase in cancer. (*Am J Pathol* 2000, 157:1549–1562)

Zhang et al,¹ using serial analysis of gene expression analysis, found that >500 genes were expressed in significantly higher or lower levels in colonic or pancreatic cancer when compared to normal epithelial tissue. One gene of increased expression was the α_1 subunit of the cardiac isoform of the voltage-gated L-type calcium channel (α_{1C}). L-type calcium channels mediate depolarization-dependent entry of Ca^{2+} ions across the plasma membrane into cells.² Calcium channel blockers such as nitrendipine, verapamil, and d-cis-diltiazem block L-type calcium channels.³ The L-type calcium channel is composed of five subunits, including the α_1 subunit which contains the ion conduction pore and the drug-binding sites, and several other subunits, α_2 , β , γ , and δ which primarily modify channel gating or affinity.⁴ The α_1 subunit of L-type calcium channels has three major isoforms found in skeletal muscle (α_{1S}); in cardiac muscle (α_{1C}); and in brain, pancreas, and GH3 pituitary cells (α_{1D}).⁵ This channel is also found in nonexcitable cells⁶ where it participates in hormone signaling pathways.^{7–9} The immunocytochemical distribution of calcium channels in epithelial cells such as colonic mucosa has not been studied, although there is physiological evidence that calcium channels mediate calcium entry in colonic cell lines.^{10–12} Although pharmacological evidence suggests the presence of L-type calcium channels in colonic epithelial

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cells,¹² it is not clear what regulates expression of the channel. The progression of colon cancer changes the expression of numerous genes,¹³ and the increased growth in colon cancer is often associated with enhanced mitogenic signals such as epidermal growth factor and epidermal growth factor receptor^{14,15} and their downstream signaling kinase c-src.¹⁶ These signaling pathways may in turn affect the activity or expression of L-type calcium channels that are modulated by c-src.¹⁷ The aim of this study was to determine whether the mRNA expression of α_{1C} is elevated in colon cancer when assayed by reverse transcriptase-polymerase chain reaction (RT-PCR) on samples from two different patient populations. Immunocytochemical localization was assessed in colon cancer compared to normal human colonic mucosa and protein expression was measured in human carcinoma cells.

Materials and Methods

Cell Lines

All cells were grown in T75 flasks following standard culture procedures or according to American Type Culture Collection (ATCC) methods if received from this source. HT29C, T84, HCT 116, and Caco-2 cells are human colonic carcinoma cell lines. HT29C cells were cultured in Dulbecco's minimal essential medium, 10% fetal bovine serum, and 10 μ g/ml of transferrin. T84 cells were cultured in a 50:50 mixture of Dulbecco's minimal essential medium/Ham's F12 and 10% fetal bovine serum. Caco-2 cells were grown in Dulbecco's minimal essential medium with 10% fetal bovine serum and 1% nonessential amino acids. HCT 116 cells p53+/+ cells (ACT CCL247) were grown in McCoy's medium with 10% fetal bovine serum. HCT 116 p53-/- that had the p53 gene removed by homologous recombination were a gift from B. Vogelstein (Johns Hopkins University, Baltimore, MD). WI-38 (CCL 75 from ATCC) a human fetal lung fibroblast cell line was grown in Eagle's basal medium and 10% fetal bovine serum. The adult human breast fibroblast cell line 966-SK obtained from breast (CRL 1881 from ATCC) was grown in Eagle's minimal essential medium with Earle's balanced salt solution, 0.1 mmol/L of nonessential amino acids, and 10% fetal bovine serum. All culture media also contained 50 U/ml penicillin and 50 μ g/ml of streptomycin. For immunohistochemistry or some Western blot experiments as indicated, cells were grown on high-pore density polyethylene terephthalate track-etched transwells (Beckton Dickinson Falcon, Franklin Lakes, NJ) coated with collagen type IV (Sigma Chemical Co., St. Louis, MO), Vitrogen (Cohesion Technologies Inc., Paulo Alto, CA), bovine serum albumin, and fibronectin.

Primers

The primers for amplifying each PCR product were taken from the nucleotide sequence of the mRNA. All primers and sequences between them were checked using a

BLAST nucleotide search at the National Center for Biotechnology Information for specificity only of the desired transcript. For α_{1C} the forward primer TGAACAGC-GACGGGACAGTCATG bp 4650 to 4673 and the reverse primer GAACGTGGCGTAGAACTTGCCAA bp 4900 to 4923, were designed using sequence from Accession No. Z74996, to give an expected PCR product of 217 bp. The area chosen for the second primer was amino acids SQRNALSL, a sequence that is not shared by α_{1D} or α_{1S} . The amino acid sequences for the same area for α_{1D} or α_{1S} are AKNTTIAL and PKKDIVQL, respectively. Primers for the vitamin D receptor (VDR) were forward primer bp 1193 to 1215 TCCAACACACTGCCAGACGTACAT and the reverse primer was bp 1723 to 1701 ATCAGTCAG-CAGCCACTTAGGCA, were designed from Accession No. J03258 to give an expected PCR product of 530 bp. For cytokeratin 20 (CK20), the forward primer was GAG-CAGTCCAACCTCCAACTTGA bp 19 to 41 and the reverse primer was TTCTTCTGGGCCATGACTTCATA bp 503 to 481, designed from Accession No. X73502 to give an expected PCR product size of 484 bp. For hepatocyte growth factor (HGF), the forward primer was GCCT-GAAAGATATCCCGACA bp 810 to 829 and the reverse primer was TTCCATGTTCTTGTCACACA bp 1332 to 1313 designed from Accession No. M60718, to give an expected PCR product size of 551 bp. For β -actin, the forward primer was TACGCCAACACAGTGTCTGTGG bp 890 to 912 and the reverse primer was TACTCCGCT-TGCTGATCCACAT bp 1095 to 1073, designed using Accession No. AB004047, to give an expected PCR product size of 206 bp. This primer was designed to cross a 112-bp intron that gave a 318-bp band if there was genomic contamination of the sample. None of the samples showed the 318-bp band for actin.

RNA Samples, RNA and cDNA Preparation, and RT-PCR

RNA was made from biopsies of colon cancer tissue or rectal tissue and the adjacent normal mucosal tissue: IRB 85-03-05-03 for patients of Johns Hopkins University and for the Vienna patients consent from the Ethics Commission of the University of Vienna Medical School was obtained before experimentation. The grade of differentiation, the stage by TNM system, and the percentage of cancer area in the total tissue biopsy, was determined from sections made from the same tissue samples (Table 1). Tumor samples were removed immediately into TRIzol (Life Technologies, Inc., Gaithersburg, MD) then total RNA was isolated following the TRIzol protocol. Total RNA and oligo (dT) primer were preheated at 65°C for 5 minutes, and then buffer, dNTP, RNase inhibitor, and M-MLV reverse transcriptase (Life Technologies, Grand Island, NY) were added at 42°C for 2 hours. Each PCR reaction of 50 μ l was composed of 1 μ l of cDNA made from 195 ng of total RNA (group A samples) or 47 ng (group B samples), 1 $\times$ PCR buffer, pH 8.5, with a 1.5 or 2.5 mmol/L Mg^{2+} Hot Wax Bead (Invitrogen, San Diego, CA), 0.2 mmol/L dATP, dCTP, dGTP, dTTP, 2.5 units of *Taq* DNA Polymerase (Life Technologies) and 0.5 μ mol/L

Table 1. Classification of Colonic Biopsies for Group A (A1–A6) and Group B (B1–B7) Used in RT-PCR of Figure 3

Patient no.	Primary lesion	Differentiation	TNM	Cancer area
A1	Sigmoid	Poorly	T3N3M0	40%
A2	Sigmoid	Moderately	T2N0M0	70%
A3	Rectum	Well	T4N3M0	30%
A4	Sigmoid	Moderately	T3N1M0	50%
A5	Cecum	Moderately	T4N2M0	60%
A6	Sigmoid	Moderately	T3N1M0	70%
B1	Cecum	Moderately	T3N1M1	40%
B2	Sigmoid	Well	T3N0M0	50%
B3	Cecum	Moderately	T3N0M0	60%
B4	Transverse	Well	T3N1M1	50%
B5	Recto-sigmoid	Moderately	T3N0M0	45%
B6	Rectum	Well	T3N0M0	60%
B7	Ascending	Moderately	T3N0M0	50%

The biopsies had an average cancer area of 52% and were evaluated by the TNM description.

primers, except the β -actin primers which were used at 0.1 μ mol/L. The DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) was programmed for 30 (VDR, β -actin), 34 (HGF), and 35 (α_{1C} , CK20) cycles. The annealing temperatures were 55°C (HGF), 60°C (VDR), or 65°C (α_{1C} , CK20, β -actin). The annealing temperature was chosen from the T_m of the primers and the optimal Mg^{2+} and pH were determined using an optimization kit (Invitrogen). Only one band of the expected size for each PCR product was found. Each PCR product was extracted from the gel and sequenced by ABR PRISM 377 DNA sequencer (Perkin-Elmer). All sequences matched with expected sequences. Water blank and non-RT samples were used as negative control. Only RT-PCR with negative controls that did not show contaminating bands were used for data analysis. For simplicity these controls are not shown in the Figures.

The amount of total RNA and PCR cycles to be used in this semiquantitative RT-PCR was determined by estimating the linear portion of the RT-PCR cycle by using increasing amounts of cDNA or increasing numbers of cycles. To estimate the linear range of the RT-PCR we used the human colon cancer cell line Caco-2 for amplification of α_{1C} , CK20, β -actin, and VDR. We used the human fibroblast cell line WI-38 for amplification of HGF. Amplified RT-PCR products were separated by electrophoresis on 2% agarose gel and were stained with ethidium bromide. The area and intensity of the gel bands were determined from scans of photographs of gels done at the same time and under same conditions using Scion Image (Scion Corporation, Frederick, MD).

Statistics

The data shown are means $\pm$ SD. A paired *t*-test was used to compare the mean values. A *P* value of <0.05 was considered statistically significant.

Immunocytochemistry

Specimens of human colon cancer obtained from eight cancer patients and normal colonic mucosa obtained from three patients without colon cancer were graded by pathological examination. Biopsy sections were heated

to melt the paraffin, then soaked in xylenes, followed by ethanol descending, then rinsed in phosphate-buffered saline (PBS) followed by 0.1% Tween 20 in PBS, and ending with a PBS wash. Sections were heated in a 10 mmol/L sodium citrate buffer (adjusted to pH 6.0) to unmask the antigen. Sections were washed with PBS, then blocked with PBS containing 5% normal goat serum and 0.3% Triton X-100. T84 cells or Caco-2 cells were grown on coated transwells, then washed with PBS, and fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 30 minutes on ice. The calcium channel α_{1C} subunit antibody used for immunohistochemistry was obtained from Alomone Labs Ltd. (Jerusalem, Israel). This affinity-purified, rabbit polyclonal antibody was raised against a peptide made from amino acids 818 to 835 (TTKINMDDLQPSNEDKS) of rat α_{1C} subunit and the corresponding amino acids in the human α_{1C} subunit are ATKINMDDLQPNENEDKS. A BLAST amino acid search at the National Center for Biotechnology Information site revealed that this amino acid sequence was specific for α_{1C} and a similar sequence was not found in any other voltage-gated calcium channel including α_{1D} , α_{1S} , N, R, or P/Q types. The primary antibody was diluted 1:20 or 1:50 with 0.1% bovine serum albumin and 0.01% sodium azide in PBS. To determine specificity of staining, 1 μ gm of the antigenic peptide was added per μ gm of primary antibody according to the directions of the supplier. After washing with PBS, the section was incubated with the secondary antibody, Cy3-goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 or 1:200 with PBS. Sections were then washed with PBS then incubated with the nuclear stain Hoechst 33324 (Molecular Probes, Eugene, OR) diluted 1:1000 with PBS. Double-fluorescence sections were analyzed with the laser-scanning confocal microscope (LSM 410; Carl Zeiss, Oberkochen, Germany).

Western Blot

The affinity-purified, rabbit polyclonal antibody against the α_{1C} subunit and the antigenic fusion protein that were used for Western blotting were obtained from Alomone Labs Ltd. This antibody was raised against the N terminal amino acids (1 to 46) MLRAL VQPAT PAYQP LPSHL

Figure 1. A and B: Estimation of the linear range of RT-PCR reaction for α_{1C} . The correlation between intensity of RT-PCR bands and number of PCR cycles (**A, left**). PCR reactions that contained the cDNA template made from 200 ng of total RNA were amplified for 31 to 44 cycles. The linear range is between 33 to 37 cycles. The correlation between intensity of PCR bands and original amount of total RNA (**B, right**).

SAETE STCKG TVVHE AQLNH FYIAP G of the cardiac α_{1C} subunit. Rat heart was used as a positive control tissue. Whole rat heart was quickly homogenized at 4°C with a polytron PT10 (Kinematica AG, Switzerland) in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS) containing 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L β -glycerol phosphate, 1 mmol/L L-phenylalanine, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and the following protease inhibitors: pepstatin A, 2 μ g/ml; leupeptin, 10 μ g/ml; aprotinin, 10 μ g/ml; elastinal, 2 μ g/ml; benzamidine, 0.5 mg/ml; calpain inhibitor peptide, 10 μ g/ml; and 1 mmol/L phenylmethane-sulfonyl fluoride, 0.4 mmol/L iodoacetic acid, 2.5 mmol/L phenanthroline, and 0.1 mmol/L *N*-tosyl-L-phenylalanine chloromethyl ketone (all from Sigma Chemical Co.). The homogenate was lysed on ice for 1 hour and centrifuged at $15,000 \times g$ for 20 minutes. The protein in the supernatant was quantified with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and stored at -80°C . The cultured cells were scraped into the RIPA lysis buffer and homogenized by passing through a 26-gauge needle 15 times. The homogenate was lysed on ice for 1 hour and centrifuged at $2,000 \times g$ for 20 minutes. The supernatant protein was quantified and stored as described above. The cell lysates, 100 μ g of heart, T84, or HCT cells, were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA) and incubated at room temperature for 60 minutes. After separating by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene sulfonyl fluoride membranes (Bio-Rad) or supported nitrocellulose membranes in Tris/glycine transfer buffer (Bio-Rad) containing 5% methanol and

0.014% sodium dodecyl sulfate. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 for 1 hour at room temperature and then were incubated with primary antibody at 3 μ g/ml diluted with blocking buffer overnight. The membrane was washed three times for 10 minutes each with Tris-buffered saline containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Life Science, Arlington Heights, IL) diluted 1:5,000 or 1:10,000 with blocking buffer for 1 hour at room temperature. After washing, blots were visualized by the Supersignal West Dura extended duration substrate (Pierce). The fusion protein used to generate this antibody was pre-incubated with primary antibody at 15 μ g/ml to determine the specificity of the labeling.

Results

Verification of Quantitative RT-PCR

The correlation between intensity of PCR bands and PCR cycles using α_{1C} primer is demonstrated in Figure 1A. This figure indicates that a linear range of the RT-PCR is achieved between 33 cycles and 37 cycles, therefore 33 cycles was chosen as the semiquantitative range. Figure 1B indicates the correlation between the intensity of the RT-PCR bands and amount of total RNA added to make the cDNA that was used in the reactions. A plateau phase was reached at 400 ng of total RNA therefore <200 ng of RNA was chosen for making the cDNA per PCR reaction. This correlation was performed for all of the primer pairs and similar results were obtained.

Figure 2. mRNA expression of α_{1C} , β -actin, CK20, HGF, and VDR by RT-PCR in colon cancer cell lines (Caco-2, T84, and HT29) and fibroblast cell lines (WI-38 and 966-Sk). RT-PCR was performed for 40 cycles and cDNA template made from 200 ng of total RNA was used. mRNA was made from semiconfluent cells grown under standard condition. α_{1C} (L-type calcium channel α_{1C} subunit); VDR; CK20 (cytokeratin 20); HGF.

Specificity of mRNA Expression in Cell Lines

Biopsies of colonic tissue consist of several types of cells including epithelial and fibroblast cells, nerve ganglia, as well as endothelial and smooth muscle cells of blood vessels. The relative abundance of markers was estimated in three colon cancer cell lines (Caco-2, T84, HT29) and two fibroblast cell lines (WI-38, 966-Sk) is shown in Figure 2. Cytokeratin 20 is a marker of relatively differentiated intestinal epithelial cells.¹⁸ The distribution along the villus tip axis has been described.^{18,19} CK20 is found only in epithelia but not in fibroblasts (Figure 2), therefore we used this as a marker of differentiated epithelial cells. HGF is produced by intestinal stromal cells causing increases in intestinal epithelial cell proliferation.²⁰ HGF may be a factor in the pathogenesis of cancer²¹ and HGF increased in the serum of patients with advanced colon cancer.²² HGF was verified as a fibroblast marker using this RT-PCR as it was not expressed in colon cells, but was abundantly expressed in the two fibroblast cell lines (Figure 2). By estimating the fibroblast compartment we could determine whether increases in calcium channel mRNA might correlate to changes in fibroblasts or epithelial cells. Fibroblasts were previously reported to have expression of α_{1C} .²³ mRNA expression of α_{1C} was present in colon cells and in the fibroblast cells (Figure 2). The mRNA of VDR is expressed in both fibroblasts and colonic epithelial cells when measured by RT-PCR (Figure 2). VDR was previously reported to be

increased in colon polyps and mid-stage tumors where the protein was found mainly to be expressed in epithelial cells.²⁴ β -actin was expressed in both epithelial and fibroblast cells in equal abundance (Figure 2). β -actin was used to standardize the amount of RNA used in each RT-PCR reaction. Because the amount of nerves and blood vessels were low, markers for these tissue types were not used.

mRNA Expression in Colon Cancer and Normal Adjacent Colon

To compare the mRNA expression in the paired colon/rectum cancer biopsies with the biopsies of adjacent tissue, we designed primers to α_{1C} across exon 41, to contain the TAG sequence used in the reported SAGE analysis.¹ Figure 3 shows the results of mRNA expression from Austrian patients (group A) and in United States patients (group B) for the chosen markers α_{1C} , VDR, CK20, HGF, and β -actin. Table 2 shows that the mRNA expression of α_{1C} alone or the α_{1C}/β -actin ratio was significantly higher in colon cancer compared to normal tissue in group A alone, in group B alone, or in groups A and B taken together (total). The $\alpha_{1C}/CK20$ ratio was also elevated in cancer tissue from group B alone or groups A and B together (total), relative to control tissues. The expression of CK20 was previously reported¹ to be lower in colon cancer and this was found again in our analysis of group A, but in group B this marker was more variable then in group A and did not show a statistical decrease. Only the two most advanced tumors of group A, T3N2M0 and T4N2M0, showed decreased CK20 suggesting that this is only a useful marker of colon cancer progression if the samples are in later stages of tumor progression. In this study, the expression of HGF mRNA was statistically increased in five of six tumor samples from group A compared to adjacent normal mucosa. Although there was also an increase in HGF in five of seven of the tumor samples from group B, the increase was not statistically different. HGF mRNA was increased in group A samples that had a greater node index than the biopsies taken from patients in group B suggesting that HGF may be an indicator of lymph node invasion. We found that VDR mRNA was elevated in cancer from group A when compared to the adjacent mucosa, but these changes were not statistically significant because of the variability in the level of VDR in the samples. However in five of six tumor samples VDR is increased compared to the adjacent normal tissue. Likewise VDR mRNA in group B United States patients was not statistically increased in tumor samples compared to normal adjacent mucosal samples. However, VDR was elevated in the two late-stage tumor samples compared to normal colon samples from these patients. The mRNA for VDR had the greatest variation of any marker measured. There was no significant difference in the β -actin marker between cancer and normal control in either group.

Figure 3. A and B: mRNA expression of α_{1C} , β -actin, CK20, HGF, and VDR by RT-PCR in colon cancer and adjacent normal mucosa. **Lanes A1–A6:** colon cancer (T) and adjacent normal mucosa (N) in six Austrian colon cancer biopsies (group A). **Lanes B1–B7:** seven United States colon cancer patients (group B). **Left lane,** marker: 100-bp DNA ladder (Life Technologies, Inc.). PCR products were resolved on a 2% agarose gel.

Immunocytochemistry of Intact Human Colon

The pattern of staining and localization in normal human colon, and rat or mouse colon (not shown) were the same. Because the staining was completely displaced by the antigen peptide in all three species, we concluded that the staining was specific to α_{1C} . There was no staining of the secondary antibody alone (not shown). Figure 4 shows the immunolocalization of α_{1C} in normal human

colonic tissues. The most intense staining of α_{1C} was localized to the colonic epithelial cells as shown by the intense red staining in this layer compared to the stromal layer which is mainly green as a result of the nuclear stain (Figure 4A). The red fluorescent staining of the secondary antibody was negligible, when the primary antibody to the calcium channel was displaced by peptide (Figure 4B). The epithelial layer can clearly be distinguished by the single row of regular nuclei (green) lining the crypts

Table 2. Compilation of Average Intensity of RT-PCR Bands in Colon Cancer and Adjacent Normal Mucosa in Group A, Group B, or Groups A and B together (Total)

	Group A (n = 6)		Group B (n = 7)		Total (n = 13)	
	Cancer	Normal	Cancer	Normal	Cancer	Normal
α_{1C}	1203 $\pm$ 360*	657 $\pm$ 607	1291 $\pm$ 537*	707 $\pm$ 343	1251 $\pm$ 447 [†]	684 $\pm$ 462
β actin	2124 $\pm$ 91	2115 $\pm$ 228	1391 $\pm$ 217	1404 $\pm$ 343	1729 $\pm$ 414	1732 $\pm$ 465
CK20	1168 $\pm$ 432	768 $\pm$ 487	1221 $\pm$ 162*	1473 $\pm$ 158	1197 $\pm$ 302	1148 $\pm$ 495
HGF	884 $\pm$ 180*	533 $\pm$ 175	633 $\pm$ 176	548 $\pm$ 283	749 $\pm$ 214 [†]	541 $\pm$ 230
VDR	852 $\pm$ 645	553 $\pm$ 530	959 $\pm$ 638	863 $\pm$ 631	910 $\pm$ 616	720 $\pm$ 585
α_{1C}/β actin ratio	0.567 $\pm$ 0.172*	0.292 $\pm$ 0.230	0.939 $\pm$ 0.386*	0.525 $\pm$ 0.282	0.767 $\pm$ 0.352 [†]	0.418 $\pm$ 0.276
$\alpha_{1C}/CK20$ ratio	1.176 $\pm$ 0.631	1.057 $\pm$ 0.898	1.086 $\pm$ 0.512*	0.481 $\pm$ 0.237	1.128 $\pm$ 0.547	0.747 $\pm$ 0.673

The mRNA of the calcium channel was elevated in colon cancer compared to normal adjacent mucosa regardless of the sample source (University of Vienna which was group A or Johns Hopkins University patients which was group B). In addition the mRNA of the calcium channel was elevated if it was standardized to actin. The marker for fibroblasts was statistically elevated in samples from group A suggesting that there was increased fibroblast content in these cancer biopsies, but this was not seen in the samples from group B. The marker for colonic surface cells CK20 showed a statistical decrease in colon cancer biopsies in group B compared to their normal controls suggesting the surface epithelial cells were less abundant in these samples. This difference was not found in samples from group A. VDR showed an upward trend in both sets of colon cancer samples but this was not statistically significant. Values are means $\pm$ SD.

* $P < 0.05$.

[†] $P < 0.01$ compared with normal mucosa.

Figure 4. A–G: Immunocytochemical localization of α_{1C} in normal human colonic mucosa. The red fluorescence stain from the Cy3-conjugated secondary antibody indicates the α_{1C} subunit. Nuclei are stained with Hoechst 33342 and appear green. **A:** The α_{1C} subunit is shown as red staining that was detected in all epithelial cells of normal human colonic tissue. **B:** There was no red staining for the calcium channel α_{1C} subunit when the antigenic peptide was added with the primary antibody. The nuclei are stained green. **C:** The staining of surface epithelial cells was mainly in the apical membrane of the cells. There was also diffuse staining at the basolateral membrane of the cells below the level of the nuclei (green). **D:** In the mid-crypt region the mucous cells were stained at the lateral and basal poles. The staining at the basolateral aspect in these cells was in the same plane as the nuclei. **E:** Staining at the bottom of the crypt was similar to that of mid-crypt but some cells were very brightly stained around the nuclear region. Other cell types also showed specific staining for the calcium channel in sections from three different samples of normal colonic tissue. **F:** There was staining in the parasympathetic ganglion in colonic smooth muscle. **G:** Endothelial cells of blood vessels were strongly stained. **H:** Colonic smooth muscle was stained. **C** and **D:** Fibroblasts showed diffuse staining over the cytoplasm.

Figure 5. A-F: Immunocytochemical localization of α_{1C} in colon cancer. Shown are three different colon cancers from three of the eight different biopsies examined. The red fluorescence stain from the Cy3-conjugated secondary antibody indicates the α_{1C} subunit. Nuclei are stained with Hoechst 33342 and appear green. Low- (A) and high-power (B) magnification images of a well-differentiated T2N0M0 sigmoid adenocarcinoma. Although the crypts seemed normal in architecture at low power, under high-power magnification the cells appear small and multilayered. The red stain for the α_{1C} subunit of the calcium channel appears to be at all aspects of the cells. This staining pattern was similar to that seen in a few cells in the lower portion of the normal colonic crypt. Low- (C) and high-power magnification (D) images show an example of moderate-strong staining for α_{1C} in a moderately differentiated T2N0M0 adenocarcinoma of the rectum. Low-power (E) and high-power (F) magnification images show weak staining for α_{1C} in one area of a poorly differentiated T3N2M0 sigmoid adenocarcinoma.

Figure 6. A–F: Immunocytochemical localization of α_{1C} in confluent cultures of T84 and Caco-2 cells grown on coated transwells 2-weeks postconfluent. The red fluorescence stain from the Cy3-conjugated secondary antibody indicates the α_{1C} subunit. The nuclei are stained with Hoechst 33342 and appear green. **A** and **B:** The red staining for α_{1C} is in the lateral, basal, and apical membranes of T84 cells. **C:** There was no red staining for the calcium channel α_{1C} subunit when the antigenic peptide was added with the primary antibody. The nuclei are stained green. **D** and **E:** Caco-2 cells have sharper lateral membrane localization and more staining on the apical membrane than T84 cells. **F:** Peptide displacement of staining in Caco-2 cells.

(Figure 4B). Both the absorptive, surface epithelial cells (Figure 4C) and crypt mucus-secreting cells (Figure 4D) showed strong staining at the cell membranes, but staining at the apical aspect of the surface cells (Figure 4C) was much stronger and reached deeper into the cytoplasm $\sim 10 \mu$ m, than the staining of mucus secreting cells (Figure 4D). There was no staining around the nuclei or above the nuclei in surface epithelial cells. The mucus secreting cells in the mid-crypt region, which have nuclei at the basal aspect of the cell, had moderate diffuse stain for the calcium channel over the nuclei. The staining of deep crypt cells was stronger than that of the mucus

secreting cells with more staining at the apical membrane and a greater proportion of the staining over the nuclei than in the mid-crypt cells (Figure 4E). The parasympathetic ganglionic nerves were moderately stained (Figure 4F). Strong staining of endothelial cells of blood vessels was also evident (Figure 4G). There was also staining in the smooth muscle (Figure 4H) and stromal fibroblast cells (Figure 4, C and D). These findings were the same in all of the normal colonic sections from three patients.

We investigated the immunohistochemical staining of α_{1C} in colon cancer biopsies from eight patients shown in Table 3, three of which are shown in Figure 5. Figure 5, A

Figure 7. A–F: A comparison of the immunocytochemical localization of α_{1C} in confluent and nonconfluent T84 cells. The red fluorescence stain from the Cy3-conjugated secondary antibody indicates the α_{1C} subunit. The nuclei are stained with Hoechst 33342 and appear green. **A–C:** Low-level red staining was detected in the lateral, basal, and apical membranes of confluent T84 cells. **B** and **C:** One cell with two nuclei has markedly intense staining compared to the nondividing cells. **D** and **E:** Intense staining is shown for nonconfluent cells that are spread out and flat. In addition to the very intense staining the staining appears localized throughout the cell cytoplasm. Because the cells are flat it is difficult to determine whether the distribution is in the membrane. Compare the cell height in the XZ plane of nonconfluent cells (**E**) with the greater height of the confluent cells shown in the XZ plane (**C**). **F:** Peptide displacement of the nonconfluent cells indicates that the intense staining is completely displaced by the antigenic peptide.

Figure 8. Western blot of rat heart, T84 cells, and HCT cells. **A:** Lanes 1 and 2 show rat heart, lanes 3 and 4 are T84 cells, and lanes 5 and 6 are HCT cells with and without p53, respectively. Lanes 2 and 4 show that the protein to which the antibody (an affinity-purified rabbit polyclonal antibody to the N terminal of α_{1C}) was raised, completely displaces the bands near 200 kD. **B:** Homogenates from six cultures of T84 cells grown 30 to 50% nonconfluent grown on plastic (lanes 4–6) versus cells 1 week postconfluent grown on plastic (lanes 1–3). There is more calcium channel protein when cells are nonconfluent and more rapidly growing (without tight junctions) than in cells that are in a low growth phase. The internal standard glyceraldehyde-3-phosphate dehydrogenase shows that less protein was added in the last three lanes compared to the first three lanes. The density/area of the bands for α_{1C} in lanes 1–3 are 126 ± 8 (mean $\pm$ SD) compared to lanes 4–6, which is 197 ± 22 (mean $\pm$ SD). **C:** Homogenates from three cultures of T84 cells grown on coated transwells 2 weeks postconfluent (lane 1) compared to cells grown 1 week postconfluent grown on plastic flasks (lane 2) and cells grown to 50% confluence in a flask (lane 3). The internal standard glyceraldehyde-3-phosphate dehydrogenase shows that nearly equal loading of the lanes was achieved.

and B, shows strong staining for α_{1C} in a well-differentiated adenocarcinoma (T2N0M0). Figure 5, C and D, shows an example of moderate staining for calcium channels in a moderately-differentiated adenocarcinoma

(T2N0M0). Figure 5, E and F, shows weak staining in an area of a poorly differentiated adenocarcinoma which had poor gland formation (T3N2M0). Staining in other tumors (not shown) staged as T2N0M0, T3N1M0, T3N2M0, T4N2M0, and T4N2M0 was moderate-strong to strong. All of the cancer cells showed a major distribution of α_{1C} in the cell membrane but some was also located near the nucleus. There did not seem to be any difference in the expression of the α_{1C} protein when the stage of the tumor was compared by the TNM system except the lower expression in one cancer (Figure 5, C and D). Fibroblast cells and smooth muscle cells were stained as in control cells (not shown).

Immunocytochemistry of Colonic Cell Lines

Immunohistochemical staining of Caco-2 and T84 cells grown as polarized monolayers on coated transwells 2 to 3 weeks postconfluent were compared to the same cells grown under nonconfluent conditions on coated transwells (15 to 20% confluent). As seen in Figure 6, A, B, D, and E, the cellular distribution of α_{1C} staining (red) in confluent T84 and Caco-2 cells is in both the basolateral and apical membranes. Caco-2 cells seem to have a more apical distribution than T84 cells (Figure 6E compared to Figure 6B). Figure 7 compares the intensity of staining in nonconfluent T84 cells compared to 2-week postconfluent T84 cells. The staining in the nonconfluent cells is very intense (Figure 7D) and appears to be in part in the cytoplasm (Figure 7E), however this is difficult to determine because the cells are very flat compared to the confluent cells shown in (Figure 7C). Of particular interest is the dividing cell in the confluent cultures (Figure 7, B and C). The cell clearly has two nuclei and the staining of α_{1C} is much more intense than that of nearby cells.

Western Blot

Western blotting of the human colon carcinoma T84 and HCT 116 cell lines revealed a band near 220 kD (Figure 8A, lanes 3, 5, and 6) that has a slightly higher molecular weight than the major band for rat heart (Figure 8A, lane 1). The protein to which this antibody was raised displaced the band in both heart and T84 cells (Figure 8A, lanes 2, and 4) confirming that this was indeed the α_{1C} subunit of the L-type calcium channel. The level of α_{1C} subunit was not a function of p53 expression (Figure 8A, lane 5 versus lane 6), nor was it increased by cAMP or decreased by indomethacin (not shown). When cells were grown as 30 to 50% nonconfluent cultures before making a whole-cell homogenate, the amount of α_{1C} subunit per total cell protein run on the gel or normalized to glyceraldehyde-3-phosphate dehydrogenase (Figure 8, bottom), was increased (Figure 8B, lanes 4 to 6) compared to cells that had been confluent for >1 week (Figure 8B, lanes 1 to 3). When the amount of α_{1C} was compared to cells grown 2-weeks postconfluent on transwells (Figure 8C, lane 1), compared to cells grown in flasks for 2-weeks postconfluent (Figure 8C, lane 2) or

Table 3. Classification of Colon Biopsies Analyzed by Immunohistochemistry, Some of Which Appear in Figure 5

Patient no.	Primary lesion	Differentiation	TNM stage	Staining of α_{1C}	
1	Sigmoid	Poor	T3N3M0	Weak	Patchy
2	Sigmoid	Moderate	T2N0M0	Moderate	Diffuse
3	Rectum	Well	T4N4M0	Strong	Diffuse
4	Cecum	Moderate	T4N2M0	Strong	Diffuse
5	Sigmoid	Moderate	T3N1M0	Moderate	Diffuse
6	Rectum	Moderate	T2N0M0	Moderate	Diffuse
7	Ascending	Moderate	T3N2M0	Strong	Diffuse
8	Sigmoid	Well	T2N0M0	Strong	Diffuse

50% confluent flask (Figure 8C, lane 3), the latter condition showed the greatest increase in channel protein.

Discussion

The mRNA expression of the α_{1C} subunit of the calcium channel was statistically increased in colon cancer compared to adjacent mucosa in biopsies obtained from two different countries. This increase in calcium channel mRNA was present in all stages of cancer from T2N0M0 to T4N2M0, although the increases appeared larger in more advanced tumors. This increase in mRNA α_{1C} subunit may be used as a marker of transformation or proliferation in biopsy samples. Colon cancer cells that have an increase in mRNA of the calcium channel may have increased proliferation that is enhanced by epidermal growth factor as reported for other cell types.⁹ Because epidermal growth factor and epidermal growth factor receptors are increased in colon cancer, and epidermal growth factor results in signaling via src kinase,¹⁶ this pathway may enhance calcium influx because calcium channels show increased signaling via src kinase.¹⁷

Staining on the apical membrane of columnar absorptive cells on the surface of human colon mucosa was stronger for α_{1C} than mucus-secreting cells in the crypt. Furthermore, the staining on the apical aspect of surface cells was stronger than basolateral staining. The staining at the apical aspect of the surface cells indicates that calcium channels are distributed in the plasma membrane where they could mediate calcium entry, but the channels are also found below the surface of cell extending approximately 10 μ m below the apical surface extending below the terminal web. This distribution below the surface of the plasma membrane suggests that these channels may be recirculated from the plasma membrane in a subapical pool. Staining in the human colonic crypt cells is mainly at the basal and lateral membranes. The expression of α_{1C} protein in colon cancer appears to be increased per mass of tissue in part because of the reduced size of the cells, which increases the surface to volume ratio. Cancer biopsies show that the calcium channel is distributed more over the nuclei than over the apical or basolateral parts of the cell, a distribution similar to that found in the cells of the crypt proliferation zone. Immunocytochemistry of T84 cells that are rapidly growing shows less membrane association and more intracellular distribution, whereas confluent cells show the calcium channel is distributed primarily in the plasma

membranes. Dividing T84 cells have an increased intensity of staining that may be reflected in the colon cancer cells.

Increases in intracellular calcium in most cell types are mediated by the release of calcium from the endoplasmic reticulum via inositol 1,4,5-trisphosphate (IP3) and influx via voltage-dependent calcium channels, receptor-operated calcium channels, and/or store-operated calcium channels. L-type calcium channels have been identified previously in HT29 colon cancer cells¹⁰ and neuroendocrine cell lines²⁵ using the patch-clamp technique. In colonic cells, acetylcholine or ATP mediate both calcium influx and release of calcium from internal stores.^{11,12} Blockers of calcium channels inhibit calcium influx into colon cells.¹² The apical distribution of this channel in colonic surface cells suggests that these channels might also participate in transepithelial calcium absorption in the colon, however a substantial calcium absorption in this segment occurs only after resection of the small bowel.²⁶ Increased calcium stimulates prostaglandin synthesis by COX 1 and COX 2²⁷ an enzyme that is elevated in colon cancer.²⁸ We have shown α_{1C} subunit protein is present in colonic T84 and HCT epithelial cells by both Western blotting and immunocytochemistry. The abundance of the calcium channel protein is not altered by cAMP or p53, but is increased in nonconfluent cells compared to confluent cultures (Figure 8, B and C) suggesting that decreased tight junctions during growth may increase the expression of this protein. However, these channels do not seem to contribute to cell growth itself, because the L-type calcium channel agonist BAYK 8644 does not increase [³H]thymidine incorporation or cell number. Both the agonist and antagonist inhibit growth nonspecifically only at μ mol/L concentrations that are well above their relative affinities on L-type calcium channels (data not shown).

HGF/scatter factor acts through c-Met tyrosine kinase to increase cell motility by disrupting cell-cell junctions thereby allowing cells to disperse. When both groups were combined, HGF mRNA was still significantly increased in colon cancer compared to normal adjacent tissue, suggesting that like the α_{1C} calcium channel, increased mRNA of HGF is also a marker of cancer but the difference between tumor and normal is not as large as that of α_{1C} . This study confirms previous work that found HGF greater in cancer tissue²² compared to normal mucosa. Increases in mRNA for HGF in early stage tumor may suggest a transformation of the stromal com-

partment early in tumor progression. Because the c-Met receptor gene is also increased in colorectal tumors,²¹ there appears a synergistic interaction between the fibroblast and epithelial compartment that would result in increased scattering of epithelial cells.

Cytokeratins are intermediate filaments that are thought to maintain the polarized integrity of intestinal cells by attaching at desmosomes to maintain cell-cell adhesion. CK20 is a major protein in mature enterocytes and goblet cells¹⁹ and was reported to be decreased in colon cancer¹ but is also decreased in the adjacent mucosa from colon cancer patients.²⁴ We found variable levels of CK20 mRNA in colon cancer samples compared to samples of normal mucosa from the same patient in group A, but group B had a small statistical decrease in CK20 mRNA in cancer tissue compared to the normal adjacent mucosa. A decrease in CK20 would support the view that tumors have fewer differentiated cells than normal colon tissue.

Low levels of vitamin D have been implicated as a risk for colon cancer in men.²⁹ Vitamin D inhibits proliferation of colon cancer cells by arresting cells in the G₀ phase of cell cycle by a direct action on p21/WAF1/CIP1.³⁰ Some vitamin D analogs like EB 1089 cause less calcemia when compared to the naturally occurring active analog 1 α ,25-dihydroxyvitamin D₃ (1,25 D₃), and are more effective in arresting cell growth than the parent compound 1,25 D₃.³¹ Colonocytes have an altered metabolism of vitamin D that is differentiation-dependent.³² The nuclear VDR increases transcription of numerous genes, some of which are involved in differentiation.³³ It was previously reported that VDR protein expression increased in early cancer stages in both tumors and adjacent mucosa (T2-T3) when compared to the normal mucosa from non-cancer patients,²⁴ but in advanced cancer (T3), VDR decreases when compared to normal mucosa from non-cancer patients.²⁴ This study indicates that any increases in VDR mRNA in tumors were not statistically different. Because the amount of VDR is regulated by 1,25 D₃,³⁴ it is possible that any association of VDR with colon cancer may be masked by dietary factors, including calcium intake and serum levels of 1,25 D₃.

In summary we conclude that the increase in mRNA of α_1 subunit of the cardiac isoform of the L-type calcium channel may be a useful marker of colon cancer compared to other markers because the increase is large and this increase can be documented on small samples using a simple semiquantitative RT-PCR. The α_1 subunit is very abundant when T84 cells are nonconfluent or dividing compared to cells that are confluent when both immunocytochemistry and Western blots are compared. We infer from this that cancer cells may also have more α_{1C} protein because these cells are more likely to be in a state of cell division.

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References

1. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, Kinzler KW: Gene expression profiles in normal and cancer cells. *Science* 1997, 276:1268–1272
2. Nowycky MC, Fox AP, Tsien RW: Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 1985, 316: 440–449
3. Henry PD: Comparative pharmacology of calcium antagonists: nifedipine, verapamil and diltiazem. *Am J Cardiol* 1981, 46(Suppl 6):S1047–S1058
4. Catterall WA: Structure and function of voltage-gated ion channels. *Annu Rev Biochem* 1995, 64:493–531
5. Perez-Reyes E, Schneider T: Molecular biology of calcium channels. *Kidney Int* 1995, 48(Suppl 4):S1111–S1124
6. Guggino SE, Lajeunesse D, Wagner JA, Snyder SH: Bone remodeling signaled by a dihydropyridine- and phenylalkylamine-sensitive calcium channel. *Proc Natl Acad Sci USA* 1989, 86:2957–2960
7. Yukihiro S, Posner GH, Guggino SE: Vitamin D₃ analogs stimulate calcium currents in rat osteosarcoma cells. *J Biol Chem* 1994, 269: 23889–23893
8. Barry ELR, Gesek FA, Froehner SC, Friedman PA: Multiple calcium channel transcriptions in rat osteosarcoma cells: selective activation of α_{1D} isoform by parathyroid hormone. *Proc Natl Acad Sci USA* 1995, 92:10914–10918
9. Loza J, Carpio L, Lawless G, Marzec N, Dziak RD: Role of extracellular calcium influx in EGF-induced osteoblastic cell proliferation. *Bone* 1995, 16(Suppl 4):341S–347S
10. Leipziger J, Fisher K-G, Greger R: Voltage-dependent Ca²⁺ influx in the epithelial cell line HT₂₉: simultaneous use of intracellular Ca²⁺ measurements and nystatin perforated patch-clamp technique. *Pflugers Arch* 1994, 426:427–432
11. Bou-Hanna C, Berthon B, Combettes L, Claret M, Laboisie CL: Role of calcium in carbachol- and neurotensin-induced mucin exocytosis in a human colonic goblet cell line and cross-talk with the cyclic AMP pathway. *Biochem J* 1994, 299(Suppl 2):S579–S585
12. Reinlib L, Mikkelsen R, Zahniser D, Dharmasathaporn K, Donowitz M: Carbachol-induced cytosolic free Ca²⁺ increases in T84 colonic cells seen by microfluorimetry. *Am J Physiol* 1989, 257:G950–G960
13. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. *Cell* 1990, 61:759–767
14. Khazaie K, Schirmacher V, Lichtner RB: EGF receptor in neoplasia and metastasis. *Cancer Metastasis* 1993, 12:255–274
15. Aaronson SA: Growth factors and cancer. *Science* 1991, 254:1146–1153
16. Maa M-C, Leu T-H, McCarley DJ, Schatzman RC, Parsons SJ: Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc Natl Acad Sci USA* 1995, 92:6981–6985
17. Hu X-Q, Singh N, Mukhopadhyay D, Akbarali HI: Modulation of voltage-dependent Ca²⁺ channels in rabbit colonic smooth muscle cells by c-Src and focal adhesion kinase. *J Biol Chem* 1998, 273:5337–5342
18. Calnek D, Quaroni A: Differential localization by in situ hybridization of distinct keratin mRNA species during intestinal epithelial cell development and differentiation. *Differentiation* 1993, 53:95–104
19. Moll R, Zimbelmann R, Goldschmidt MD, Keith M, Laufer J, Kasper M, Koch PJ, Franke WW: The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation* 1993, 53:75–93
20. Goke M, Kanai M, Podolsky DK: Intestinal fibroblasts regulate intestinal epithelial cell proliferation via hepatocyte growth factor. *Am J Physiol* 1998, 274:G809–G818
21. Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L, Nordlinger B, Bretti S, Bottardi S, Giordano S, Plebani M, Gespach C, Comoglio PM: Overexpression and amplification of the Met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* 1995, 1:147–154
22. Fukaura T, Miki D, Inoue T, Matsumoto K, Suzuki H: Serum hepatocyte growth factor as an index of disease status of patients with colorectal carcinoma. *Br J Cancer* 1998, 78:454–459

23. Soldatov NM: Genomic structure of human L-type Ca^{2+} channel. *Genomics* 1994, 22:77–87
24. Cross HS, Bajna E, Vises G, Genser D, Kallay E, Potzi R, Wenzl E, Wrba F, Roka R, Peterlik M: Vitamin D receptor and cytokeratin: expression may be progression indicators in human colon cancer. *Anticancer Res* 1996, 16:2333–2338
25. Glassmeier G, Strubing C, Riecken EO, Buhr H, Neuhaus P, Ahnert-Hilger G, Weidenmann B, Scherubl H: Electrophysiological properties of human carcinoid cells of the gut. *Gastroenterology* 1997, 113:90–100
26. Hylander E, Ladefoged K, Jarnum S: The importance of the colon in calcium absorption following small-intestinal resection. *Scand J Gastroenterol* 1980, 15:55–60
27. Longo WE, Panesar N, Mazuski J, Kaminski DL: Contribution of cyclooxygenase-1 and cyclooxygenase-2 to prostanoid formation by human enterocytes stimulated by calcium ionophore and inflammatory agents. *Prostaglandins Other Lipid Mediat* 1998, 56:325–329
28. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994, 107:1183–1188
29. Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED: Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* 1989, 18:1176–1178
30. Liu MMH, Cohen M, Bommakanti M, Freedman LP: Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev* 1996, 15:142–153
31. Hansen CM, Maenpaa PH: EB 1089, a novel vitamin D analog with strong antiproliferative and differentiation-inducing effects on target cells. *Biochem Pharmacol* 1997, 154:1173–1179
32. Bischof MG, Siu-Caldera ML, Weiskopf A, Vouras P, Cross HS, Peterlik M, Reddy GS: Differentiation-related pathways of 1α , 25 dihydroxy-cholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells: production of 1α , 25-dihydroxy-3epi-cholecalciferol. *Exp Cell Res* 1998, 241:194–201
33. Dusso AS, Brown AJ: Mechanism of vitamin D action and its regulation. *Am J Kidney Dis* 1998, 32:S13–S24
34. Zineb R, Zhor B, Odile W, Marthe RR: Distinct, tissue-specific regulation of vitamin D receptor in the intestine, kidney, and skin by dietary calcium and vitamin D. *Endocrinology* 1998, 139:1844–1852