

Research article

***COL11A1* in FAP polyps and in sporadic colorectal tumors**

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Abstract

Background: We previously reported that the α -1 chain of type II collagen (*COL11A1*), not normally expressed in the colon, was up-regulated in stromal fibroblasts in most sporadic colorectal carcinomas. Patients with germline mutations in the *APC* gene show, besides colonic polyposis, symptoms of stromal fibroblast involvement, which could be related to *COL11A1* expression. Most colorectal carcinomas are suggested to be a result of an activated Wnt- pathway, most often involving an inactivation of the *APC* gene or activation of β -catenin.

Methods: We used normal and polyp tissue samples from one FAP patient and a set of 37 sporadic colorectal carcinomas to find out if the up-regulation of *COL11A1* was associated with an active *APC*/ β -catenin pathway.

Results: In this study we found a statistically significant difference in *COL11A1* expression between normal tissue and adenomas from one FAP patient, and all adenomas gave evidence for an active *APC*/ β -catenin pathway. An active Wnt pathway has been suggested to involve stromal expression of *WISP-1*. We found a strong correlation between *WISP-1* and *COL11A1* expression in sporadic carcinomas.

Conclusions: Our results suggest that expression of *COL11A1* in colorectal tumors could be associated with the *APC*/ β -catenin pathway in FAP and sporadic colorectal cancer.

Background

The majority of colorectal carcinomas is sporadic. Mutations in the *APC* gene are somatically derived and prevalent in more than 80% of sporadic cases [1]. A minority of colorectal cancers is inherited. In familial adenomatous

polyposis (FAP), one allele is inactivated in germline, and the other one somatically [2,3]. In hereditary non-polyposis colorectal cancer (HNPCC) tumor progression is evoked by an inherited deficiency in one of the DNA mismatch repair genes, and a somatic mutation in the

APC gene is often a result of an increased mutation rate [4]. The APC protein normally binds to β -catenin in the cytoplasm and this leads to a rapid degradation of free β -catenin [5]. Inactivation of the *APC* gene results in a reduced degradation of β -catenin. β -catenin then accumulates in the nucleus, gets attached to Tcf-4/Lef, a transcription factor activating Wnt target genes [6,7]. β -catenin has also been suggested to activate the *WISP-1* gene, a member of the connective tissue growth factor family, signaling oncogenic events downstream [8,9].

Collagen is the major component of the interstitial extracellular matrix (ECM). ECM is known to play an active role in numerous biological processes such as cell morphogenesis, proliferation, migration, differentiation, apoptosis as well as carcinogenesis [10,11]. In a previous study we found that colonic mucosa stromal cells in the majority of colorectal cancers expressed *COL11A1*, while *COL11A1* was expressed only rarely in normal colon mucosa [12]. *COL11A1* expression was also found in sporadic adenomas although the difference between normal and adenoma samples was not statistically significant (12). However, only a limited number of polyps were available for study [12].

A mutation in the *APC* gene constitutes an important event in colorectal carcinogenesis. The Wnt pathway has been suggested to regulate events also in connective tissue, such as activation of the *WISP-1* gene. We therefore hypothesized that also the up regulation of *COL11A1* could be a consequence of an activated Wnt pathway. The hypothesis fits well with the extra-colonic manifestations in FAP, such as skeleton- eye- and mesenteric (desmoids) fibromatosis, which could result from an over-expression of collagens [13]. It is also striking that an inactivating mutation in the *COL11A1* gene involves the same organs, since affected individuals with Stickler's syndrome show skeleton and ophthalmic manifestations, such as osteochondrodysplasia, and retinal detachment and blindness [14].

In this study we investigated the possible up regulation of *COL11A1* in FAP and sporadic colorectal cancer using tissue samples from one FAP patient and patients with sporadic colorectal carcinomas.

Materials and methods

Tumors

Twenty-six normal tissue samples from a FAP patient were collected from cecum, ascending, transverse, and descending colon as well as rectum. Fourteen polyps were collected from the transverse and descending colon and rectum. The polyps were 2 mm in diameter. The FAP polyps and 37 sporadic carcinomas and 6 normal tissue samples were obtained immediately after surgery and

frozen at -70°C . Samples were collected at Umeå University Hospital and Karolinska Hospital with ethical permission according to guidelines in each hospital.

RNA and DNA preparation

Total RNA was extracted from frozen tissue by homogenizing with a power homogenizer (IKA Labor Technik) in Trizol Reagent according to manufacturer's protocol (Life Technologies) and used for RT-PCR. The RNA was treated with deoxyribonuclease I (Ambion). DNA was extracted from frozen tissue by homogenizing with a power homogenizer (IKA Labor Technik) in STAT 60 due to manufacturer's protocol (Tel-test "B") and used for PCR.

RT-PCR

Primers designed for *COL11A1*, *COL5A2*, *WISP-1* and the internal control *GAPDH* were used on templates from tumors and normal tissue for comparison of expression. Total RNA (2 μg) was reverse transcribed in a total of 40 μl reaction mix, with the following conditions: 5 mM MgCl_2 , 50 mM KCL, 10 mM Tris-HCl, pH 8.3, 1 mM dNTP's, 1 U RNase inhibitor (Perkin Elmer), 2.5 U MuLV Reverse transcriptase (Perkin Elmer). PCR reaction volume was 25 μl containing 250 ng of each primer, 250 nM of dNTP's, and 2.5 U of *ampliTaq* (Gibco, Life Technologies). cDNA was diluted 1:2.5 in water and 10 μl was used for all samples and controls. The primers used were as follows: *COL11A1* 5'-AGG AGA GTT GAG AAT TGG GAA TC-3', 5'-TGG TGA TCA GAA TCA GAA GTT CG-3, for *COL5A2* 5'-AAA TGA TGG TGC AGG AGG TC-3', 5'-ATT GCC AGC TGG ACC TTC-3', for the control *GAPDH2f* 5'-GAA GGC TGG GGC TCA TTT G-3', 5'-GAT GGC ATG GAC TGT GGT CA-3. Thermocycler (PTC 225, Mj Research) was used with an initial denaturation step of 94°C for 5 min, followed by cycles of 94°C for 45 s, 59°C for 30 s, 72°C for 30 s, and a final elongation step of 72°C for 5 min. The PCR products were amplified 25, 30, and 36 cycles for linear range and separated on a 1.5% agarose gel containing ethidium bromide, experiments were repeated three times using the optimal cycle number. Each experiment was accompanied by amplification of *GAPDH* as a control and the intensities of the cDNA bands for the PCR product were normalized to the *GAPDH* band intensities. The intensity of the bands was estimated with the Image Gauge V3.41 software (Fuji Film Science).

Sequencing

The germline mutation in exon 15 of the FAP patient was determined using Big Dye kit sequencing on an ABI sequencer (Perkin Elmer).

Protein truncation test (PTT)

A 1.3 kb fragment of the *APC* exon 15 was amplified using the *apcT7*-modified primer 15eT7(5'GGA TCC TAA TAC

GAC TCA CTA TAG GAA CAG ACC ACC ATG CTT AAA TAT TCA GAT GAG CAG TTG AA 3') and the antisense primer apc15;3 (5'GAG CCT CAT CTG TAC TTC TGC 3') [15]. Approximately 200 ng genomic DNA and 350 ng of each PCR primer were dispensed in a 50 μ l PCR reaction buffer (200 μ M dNTPs, 1.5 mM MgCl₂ and 2 U Taq polymerase). PCR conditions were 96°C for 1.30 min, followed by 35 cycles of (96°C for 30s, 60°C for 1.30 min, and 70°C for 1.30 min), and a final elongation step at 70°C for 5 min. Protein truncation test (PTT) was performed according to the manufactures instructions (Promega). Incorporation of ³⁵S-Metionin (Amersham) was used to detect the translation products after electrophoretic separation on a 5% SDS-polyacrylamid gel. The gel was then dried on a vacuum gel dryer and exposed to X-ray film (Cronex 4, Sterling Diagnostic Imaging).

LOH

Loss of the APC alleles was tested with PCR on normal colon mucosa, polyps and tumors. To a 25 μ l reaction, 100 ng of DNA, 1.5 mM MgCl₂, 200 μ M dNTPs, 0.25 μ M of flouochrome marked D5S346F, 0.25 μ M D5S346R, and 1U Taq polymerase (Perkin Elmer), was applied. The PCR program was 96°C for 2 min, followed by 40 cycles of 96°C – 30 sec, 50°C – 30 sec, 72°C – 30 sec, and a final elongation step 72°C for 3 min. The PCR products were analyzed on a sequencing gel (ABI, Perkin Elmer).

Statistical analysis

The difference between mRNA expression in normal samples and carcinomas in sporadic cases, and in normal tissue and polyps from the FAP patient was tested by a non-parametric method, Mann-Whitney U test, where $p < 0.05$ was considered systematic/statistically significant. Correlation between *COL11A1* and *WISP-1* expression was studied by linear correlation and calculated by Sperman rank order correlation coefficient. Experiments and analyses were made twice.

Results

Correlation between APC/ β -catenin pathway and *COL11A1* expression in FAP

First the APC germline mutation was determined using sequencing. It was a deletion of 4 base pairs at nt 4043 in exon 15, codon 1347, confirming the diagnosis of FAP in the patient. Twenty-six samples from normal colon tissue and 14 polyps from the FAP patient were tested for *COL11A1* expression and inactivating mutations of the APC gene. RNA extracted from fresh frozen samples from the resected colon in the FAP patient were then studied using a semi-quantitative RT-PCR. Grossly normal biopsies from caecum, ascending, transverse, descending colon, and rectum, as well as polyps from ascending and descending colon, and rectum, were analyzed. Using a three fold reproduced RT-PCR experiment

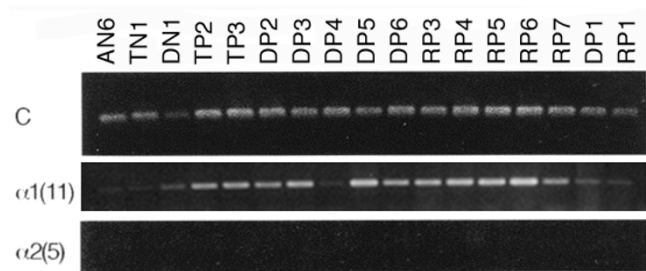


Figure 1

Example on RT-PCR comparing expression of *GAPDH* (C), *COL11A1* ($\alpha 1(11)$), *COL5A2* ($\alpha 2(5)$) in normal (N), and polyp tissues (P) from one FAP patient. AN, TN, DN, macroscopically normal tissue taken from ascending, transverse, descending colon respectively, TP, Polyp from transverse colon, DP, Polyp from descending colon, RP, Polyp from rectum.

(examples shown in figure 1), it was shown that polyps expressed *COL11A1* to a statistically significantly higher level than normal tissue samples ($p = 0.001$) (figure 2a). The relative lack of RNA made it possible only to make one northern blot experiment using a small number of samples. It was not possible to confirm the results in this experiment, since no signal for *COL11A1* was obtained for normal tissues or polyps. The FAP adenomas showed a similar level of expression as the sporadic adenomas in our previous study [12]. A comparison of the levels of *COL11A1* expression between FAP adenomas and sporadic colonic carcinomas was demonstrated in a RT-PCR experiment where FAP adenomas and sporadic colorectal tumors were run at the same time (figure 2b). The cancers expressed the RNA to a much higher degree than adenomas. In our previous study, *COL11A1* and *COL5A2* were co-expressed in sporadic colorectal cancer [12]. We therefore tested whether *COL5A2* expression was also associated with *COL11A1* in FAP adenomas. However, no expression of *COL5A2* was found in FAP normal cells or FAP adenomas (examples shown in figure 1). Normal FAP tissue where cells have one APC-allele mutated in germline did not express *COL11A1*, why we hypothesized that a complete inactivation of the wild-type allele by a second mutation was necessary for the up-regulation of *COL11A1*. The 14 FAP adenomas were therefore studied for inactivating second mutations of the wild-type allele by mutation screening using protein truncation test (PTT) and loss of heterozygosity (LOH) studies. All polyps tested had mutations and/or LOH of the wild type allele (figure 3a). Thus, all FAP samples gave evidence for an inactivated wild-type allele. This supports the idea that all polyps were established through an inactivation of the APC gene on both alleles, which triggered the ade-

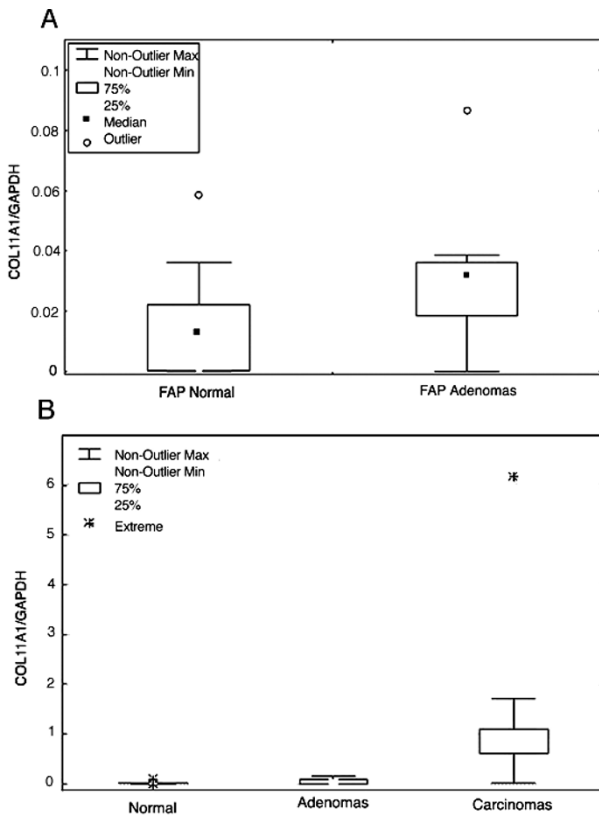


Figure 2
 Box Plots illustrating the difference between expression of *COL11A1* showing FAP normal tissue samples compared to FAP polyps (2a), and FAP polyps compared to sporadic colorectal carcinomas (2b). The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. An observation that lies more than 1.5 times the height of a box from the box is defined as *outliner* and when an observation lies more than 3 times the height of a box from the box, it is defined as an *extreme* value.

noma formation and maybe also the up-regulation of *COL11A1* in FAP.

Correlation between APC/ β -catenin pathway and *COL11A1* expression in sporadic colorectal cancer

In an attempt to relate the up-regulation of *COL11A1* expression to the inactivation on the *APC* gene in sporadic cancer, a panel of 37 sporadic colorectal carcinomas was used.

These samples were already studied for *COL11A1* expression by using RT-PCR in a previous study [12]. Mutations of the *APC* gene were searched for by using the protein truncation test (PTT) of exon 15, known to contain the majority of mutations in the so-called mutation cluster region (MCR) (figure 3b). In total 22 of 37 (59%) tumors

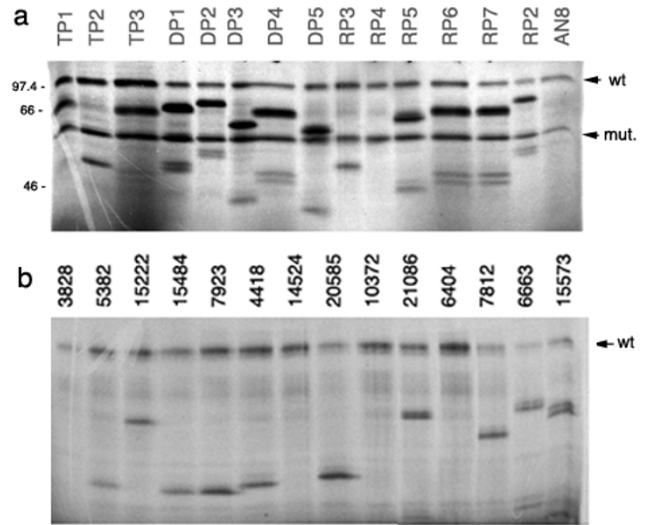


Figure 3
 Protein truncation test (PTT) on a) FAP adenoma samples, wt, wild type allele, mut, germline mutated allele, other bands indicate secondary mutations of either the wild type or the germline mutated allele. TP, polyp from transverse colon, DP, polyp from descending colon, RP, polyp from rectum, AN, normal FAP tissue taken from ascending colon. The marker CFA756 was used to approximate the size in KD (left), b) examples of sporadic colorectal carcinomas.

had a truncated mutation in exon 15. There was no correlation between *COL11A1* expression and a truncating mutation in exon 15 of the *APC* gene. However, the pathway can be activated by other mutations outside exon 15, by mutations not detected by our method, by mutations in other Wnt related genes or even epigenetic alterations. Therefore, we hypothesized that *COL11A1* might be up-regulated in a similar fashion as and together with *WISP-1*. Using the expression of *WISP-1* as an estimate of *APC*/ β -catenin activation, a study was undertaken to find out if *COL11A1* expression correlated with the expression of *WISP-1* in the sporadic colorectal tumor samples. There was a statistically significant up-regulation of *WISP-1* in sporadic carcinomas compared to the expression in normal tissue ($p < 0.002$). The expression of *COL11A1* correlated well with the up-regulation of *WISP-1* (correlation coefficient = 0.72, $p < 0.001$) in the tumors (Fig. 4). The expression of *WISP-1* in FAP samples did not differ between normal tissue and polyps ($p < 0.72$) with the method used by us.

Discussion

In this study we found a statistically significant over-expression of *COL11A1* in polyps from a patient with FAP. We have previously shown that *COL11A1* was upregulated in the majority of sporadic colorectal cancer [12]. We

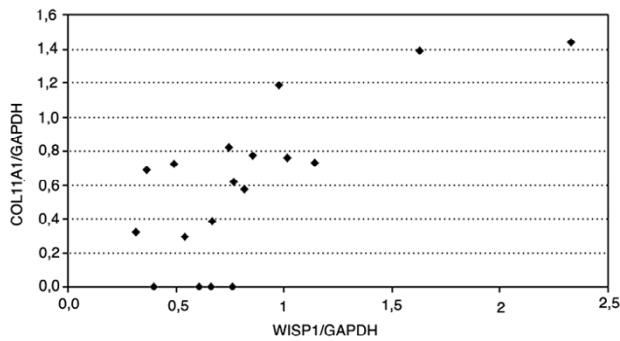


Figure 4
Diagram showing the correlation between *COL11A1* and *WISP-1* expression in sporadic colorectal carcinomas.

hypothesized that the Wnt pathway could be directly involved in the up-regulation of *COL11A1* in similarity to the extracellular molecule *WISP-1*. The Wnt pathway is involved in development of dorsal mesoderm induction and is also likely to be involved in development of the gut in humans. This is consistent with the expression of *COL11A1* in human fetal colon as well as other human developing tissues, mainly skeleton and tendon [16,17].

***COL11A1* is up-regulated in the *APC/β-catenin* pathway in FAP and sporadic colorectal cancer**

COL11A1 was up-regulated in small colorectal polyps, from one FAP patient, but to a lower degree than seen in sporadic carcinomas. This supports the hypothesis that the *APC/β-catenin* pathway could influence *COL11A1* expression in FAP. Not all sporadic colorectal carcinomas expressed *COL11A1* in our previous study [12]. There was no association between an exon 15 truncating *APC* mutation and the expression of *COL11A1* in the 37 sporadic colorectal tumors in this study. In FAP there are frequent examples of an inactivation of both *APC*-alleles in tumors, while in attenuated *APC* and sporadic colorectal cancer this is not equally frequent [18,19]. Still, the *APC/β-catenin* pathway is supposed to be involved in the vast majority of sporadic tumors, why we next used the expression of *WISP-1* as an estimate of *APC/β-catenin* pathway activation in the sporadic tumors. *WISP-1* is a member of the connective tissue growth factor family, shown to be over-expressed in 80% of colonic adenocarcinomas. It has been suggested to be activated by *β-catenin* [8], active in the Wnt pathway [20,21]. *WISP-1* was up-regulated in sporadic tumors and there was a good correlation between *COL11A1* and *WISP-1* expression in the tumors. This correlation suggests that the over-expression of *COL11A1* could be related to the *APC/β-catenin* pathway. However, some sporadic tumors with a truncating mutation in exon 15 did not show an up-regulation of the *COL11A1*, and there must be some explana-

tion for that. One possible explanation could be the RT-PCR technique used, or that RNA for some samples could have been degraded for these particular genes even though RNA was not degraded for control genes. It is also possible that there is no association with the *APC/β-catenin* pathway

FAP adenomas showed no up-regulation of *WISP-1* or *COL5A2*

WISP-1 was also expressed in normal tissue as well as in FAP, but no clear up-regulation of *WISP-1* could be determined in FAP adenomas. However, if there is an association with *COL11A1* expression, the much lower expression in adenomas may explain the lack of detectable up-regulation of *WISP-1*. The fact that *COL5A2* was not expressed in the FAP adenomas, in contrast to sporadic carcinomas, suggests that *COL5A2* could be involved in the transition from a benign to malignant state in colorectal cancer. Alternatively, the *COL5A2* (as well as *WISP-1*) could be associated with a much higher expression of *COL11A1*, as seen in sporadic colorectal cancer.

Is the mechanism of *COL11A1* transcription in FAP different from that in sporadic colorectal cancer?

In FAP there may be a direct regulation of *COL11A1* in the stromal cells since also the stromal cells have mutations in the *APC* gene. In sporadic colorectal cancer, the pathway must be activated and influence stromal cells by a somatic mutation in an epithelial cell. In the case of *WISP-1*, a model has been proposed where epithelial cells express *WISP-1* to activate the Wnt pathway in stromal cells, which answer with secreting *WISP-1*, which in turn stimulates growth. A paracrine and then an autocrine production of *WISP-1* might efficiently stimulate epithelial cells to continue unregulated proliferation and eventual tumor formation [9]. There may be a similar way for how the *APC* mutation in epithelial cells can induce a reaction in surrounding stromal fibroblasts in sporadic colorectal cancer.

Conclusion

The results from this study suggest that the up-regulation of *COL11A1* in early adenomas in FAP and in sporadic colorectal carcinomas is associated with an activated *APC/β-catenin* pathway. The expression of *COL11A1* could directly contribute to tumorigenesis in fibroblasts in FAP and explain osteomas and desmoids, or indirectly to polyp-formation and tumor progression in sporadic colorectal cancer.

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