Differentiation of tubular and villous adenomas based on Wnt pathway-related gene expression profiles

ANTHONY V. NGUYEN[1], C. GREGORY ALBERS[2] and RANDALL F. HOLCOMBE[1]

[1]Division of Hematology/Oncology and Chao Family Comprehensive Cancer Center, University of California, Irvine; [2]Division of Gastroenterology, University of California, Irvine, CA, USA

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Abstract. This study was undertaken to define whether differences in the expression of Wnt pathway components are present between normal colonic mucosa, early (tubular) adenomas and villous adenomas which have a higher malignant potential. Normal mucosa, tubular adenomas and villous adenomas were obtained from twelve patients. RNA was isolated and utilized for Wnt pathway-specific membrane array expression analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) and fluorescent immunohistochemistry (IHC) were utilized for confirmatory analyses. Fifteen Wnt pathway-related genes showed differential expression between villous adenomas and normal mucosa and villous and tubular adenomas at a significance level of $p<0.01$. Genes involved in canonical Wnt (ß-catenin) signaling with increased expression in villous adenomas included *wnt1*, *fz2*, *csnk2A2*, *pygo2*, *pygo1*, *frat2* and *myc*, the latter confirmed by qRT-PCR and IHC. Myc protein expression was confined primarily to stromal components of villous adenomas. Genes involved in non-canonical Wnt signaling with increased expression in villous adenomas included *rho-u*, *daam1*, *damm2*, *cxxc4* and *nlk*. Successive increases in the expression of *ctnnb1* (ß-catenin) from normal to tubular adenomas to villous adenomas was seen. The Wnt pathway gene expression profile can differentiate between tubular and villous adenomas. These data suggest that Wnt signaling regulation changes during the progression from normal mucosa to tubular adenomas to villous adenomas. Expression of Myc in adenoma stroma suggests a dynamic signaling network within adenomas between mucosal and stromal elements. Inhibition of the Wnt pathway may provide a novel approach for cancer prevention in patients with benign tubular adenomas.

Correspondence to: Professor Randall F. Holcombe, Division of Hematology/Oncology, University of California, Irvine Medical Center, 101 The City Drive, Building 56, Room 247, Orange, CA 92868, USA
E-mail: rholcomb@uci.edu

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Introduction

Fearon and Vogelstein (1) developed the concept that an accumulation of genetic events within colonic cells confers a growth advantage resulting in the histological progression from normal mucosa, to early and late adenomas, and eventually to carcinoma *in situ* and invasive cancer. An early event in this process is inactivation of *apc*, the gene responsible for familial polyposis, the protein product of which is central in the control of Wnt/ß-catenin signaling. While most colon cancers arise in adenomas, they occur in nearly 40% of the Western population but <5% eventually proceed to cancer (2). Those adenomas which are large and contain significant villous features are considered to have the greatest potential for malignant change (3).

Many alterations in the expression of Wnt pathway genes have been described in colon cancer (4) though the significance of upstream alterations in the expression of Wnt components were initially considered to be less significant given the downstream mutational activation of Wnt signaling related to APC inactivation and ß-catenin stabilization. Recent studies have demonstrated, however, that modulation of upstream components of the Wnt pathway can have significant effects on the strength of Wnt throughput (5,6) in colon cancer, even in the face of activating *apc* mutations. Such modulation may affect ß-catenin localization (7) and, therefore, the expression of Wnt target genes.

Since only a small proportion of adenomas progress to malignancy, and given the recognition that non-*apc* components of the Wnt pathway can modulate signaling, we evaluated the expression of multiple Wnt pathway-related genes in normal colonic mucosa and in tubular and villous adenomas. We demonstrated here that more than a dozen Wnt-related genes are differentially expressed and that the expression profiles of tubular and villous adenomas are distinct.

Materials and methods

Tissue acquisition and histology review. Institutional review board (IRB)/ethics committee approval from the University of California, Irvine IRB was obtained for this reported study. All research complies with the Declaration of Helsinki. Approximately 50 patients were identified for participation in an IRB-approved tissue acquisition study as individuals scheduled for screening or diagnostic colonoscopy at the

University of California, Irvine Medical Center. Written, informed consent was obtained from each patient prior to any sedation. Biopsies of normal mucosa and resected adenomas were obtained from twelve patients and transported to the pathology department where a small sample of tissue was allocated for research testing and immediately placed into RNA later (Applied Biosystems, Foster City, CA). Total RNA was later isolated with a Trizol reagent. No separation of mucosal and stromal elements of the biopsy samples was attempted prior to RNA isolation.

Wnt-specific membrane array. cDNA was generated from tissue-derived RNA and was analyzed with a GEArray Q Series Human Wnt Signaling Pathway Gene Array (SuperArray Bioscience, Frederick, MD) which is designed to systematically profile the expression of genes involved in and downstream of Wnt signaling. The array includes the Wnt ligands and their receptors, intracellular signaling molecules, and representative target genes. The GEArray expression analysis software was utilized for background normalization, correction for different degrees of exposure, and normalization with multiple house-keeping gene controls on each membrane.

Quantitative real-time polymerase chain reaction. Quantitative real-time PCR (qRT-PCR) was utilized to define the expression of *myc*. Specific primer sets were employed and expression was compared to actin controls in all cases. Efficiency of the *c-myc* and actin primers was equivalent. Primer pairs were obtained from Qiagen (Valencia, CA), catalog numbers QT00035406 and QT00095431 with cycling parameters as defined by the manufacturer. For qRT-PCR experiments, the relative RNA expression was calculated by the comparative threshold cycle method.

Fluorescent immunohistochemistry. Paraffin embedded tissue samples were deparaffinized with xylenes and alcohol and rehydrated. Endogenous peroxidases inhibited by incubating with H_2O_2/methanol for 30 min. Cells were then stained with monoclonal anti-c-Myc antibodies (clone ab32072, Abcam Inc., Cambridge, MA). Following counterstaining with an ABC immunohistochemistry kit (Santa Cruz Biotechnology, Santa Cruz, CA), peroxidase localization with a tyramide-fluorochrome system (Perkin Elmer, Boston, MA) was performed, and visualization was obtained by fluorescence microscopy.

Statistical analysis. Gene expression by microarray among the three tissue type groups (normal mucosa and tubular and villous adenoma) was evaluated by one-way ANOVA to define statistical differences. Comparisons between individual pairs (tubular adenoma vs. normal mucosa, villous adenoma vs. normal mucosa, villous adenoma vs. tubular adenoma) were performed via the Newman-Keuls post-test analysis. Levels of significance were recorded. To adjust for multiple comparisons, a higher stringency for the level of significance was utilized, with significance defined as $p<0.01$.

Results

Comparing gene expression by microarray, 34 genes were found to have differences in expression among normal, tubular and villous adenoma tissues at a level of significance by one-way ANOVA of $p<0.05$. However, only 15 genes demonstrated significantly different levels of expression at the $p<0.01$ level and these are depicted in Table I. All of these genes demonstrated significantly different levels of expression in villous adenomas compared to normal mucosa by the Newman-Keuls post-test at a level of $p<0.01$. Seven genes demonstrated differential expression between villous and tubular adenomas at this level of significance. On histological review, no samples were classified as tubulo-villous, or intermediate between tubular and villous adenomas.

Table I. Differentially expressed Wnt-related genes by level of statistical significance.

Gene	One-way ANOVA	Newman-Keuls post-test: Tubular vs. normal	Newman-Keuls post-test: Villous vs. normal	Newman-Keuls post-test: Villous vs. tubular
wnt1	0.0003	ns	<0.001	<0.001
damm2	0.0008	ns	<0.001	<0.001
cxxc4	0.0012	ns	<0.01	<0.01
myc	0.0045	ns	<0.01	<0.01
pygo2	0.0052	ns	<0.01	<0.05
csnk2a2	0.0054	ns	<0.01	<0.01
nlk	0.0057	ns	<0.01	<0.01
fz2	0.0059	ns	<0.01	<0.05
nkd1	0.0065	ns	<0.01	<0.01
frat2	0.0068	ns	<0.01	<0.05
foxn1	0.0077	ns	<0.01	<0.05
rhou	0.0081	ns	<0.01	<0.05
axin1	0.0089	ns	<0.01	<0.05
pygo1	0.0097	ns	<0.01	<0.05
damm1	0.01	ns	<0.01	<0.05

Values represent p-values. Genes with p-values <0.01 on one-way ANOVA testing are considered significant (ie, p>0.01, not shown); ns=not statistically significant.

The differences in expression of the four genes with the highest level of statistical significance (all with $p<0.005$), *wnt1*, *damm2*, *cxxc4* and *myc*, are depicted graphically in Fig. 1 (panels A-D). The increased expression of each of these genes in villous adenomas compared to tubular adenomas and normal colonic mucosa is readily apparent. There was no statistically significant difference between tubular adenomas and normal mucosa for any of these genes (Table I).

The gene with the highest fold increase in villous adenomas was *ctnnb1*, ß-catenin. ß-catenin was increased 2.62-fold in tubular adenomas compared to normal mucosa and 6.06-fold in villous adenomas compared to tubular adenomas. The fold increase in villous adenomas compared to normal mucosa was 15.9-fold. Because tubular adenoma expression was intermediate between normal mucosa and villous adenomas for ß-catenin, the overall level of significance by one-way ANOVA ($p<0.037$) was not as strongly significant than for the genes noted above. The expression of ß-catenin is depicted in Fig. 1, panel E.

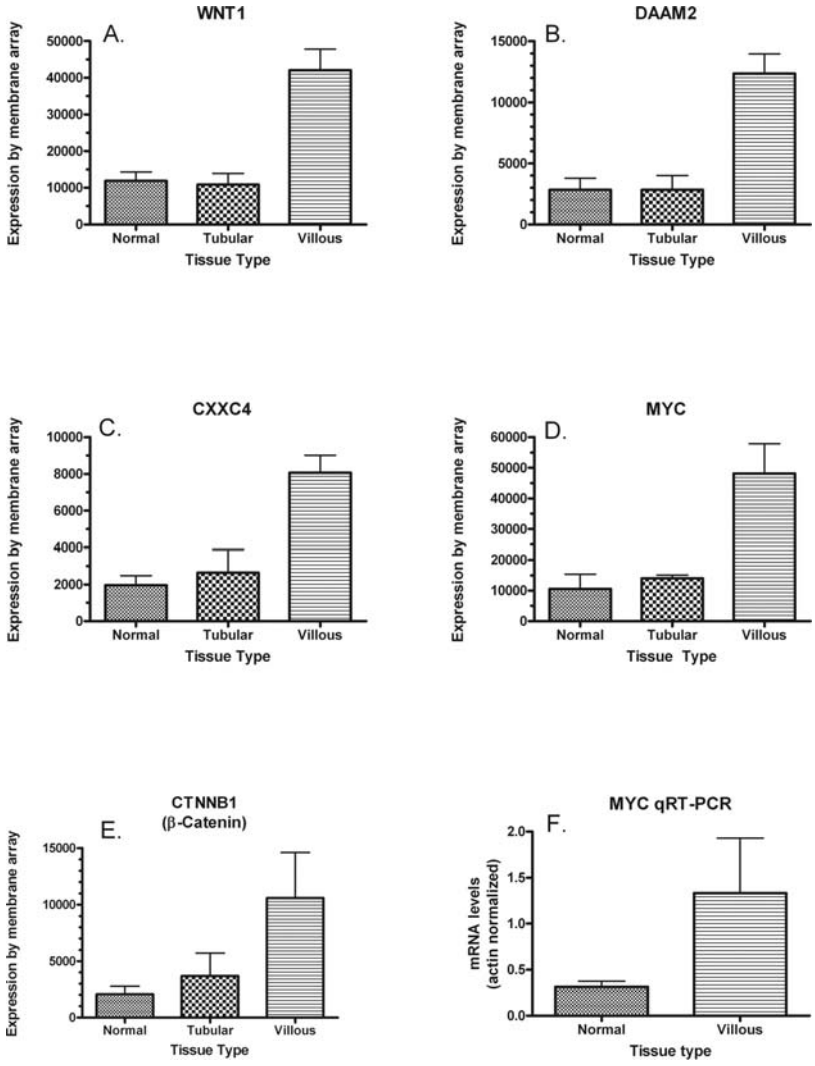

Figure 1. Expression of selected Wnt-related genes in normal colonic mucosa, tubular and villous adenomas. Expression by membrane array (panels A-E) and by quantitative real-time polymerase chain reaction (panel F).

Increased villous adenoma expression of *myc*, a well characterized Wnt pathway target gene, was confirmed by qRT-PCR. The expression in villous adenomas was 4.25-fold above expression in normal mucosa (Fig. 1, panel F). This fold increase by qRT-PCR is equivalent to the fold increase seen by microarray of 4.56. Expression of Myc protein was evaluated by fluorescence immunohistochemistry (Fig. 2) and shown to be elevated in villous adenomas compared to normal colonic mucosa and tubular adenomas. Interestingly, Myc protein expression was primarily confined to stroma components of the villous adenomas (Fig. 2, panels F and H).

Discussion

Even though Wnt pathway-activating mutations in *apc* are considered to be early events in colon carcinogenesis, occurring prior to the formation of early adenomas (1), we found significant differences in the pattern of Wnt pathway gene expression between tubular and villous adenomas. Villous adenomas are considered to have a much higher potential for malignant transformation than tubular adenomas (2). Our results suggest that the degree of Wnt pathway activation is distinct in these two types of adenomas, implying that factors

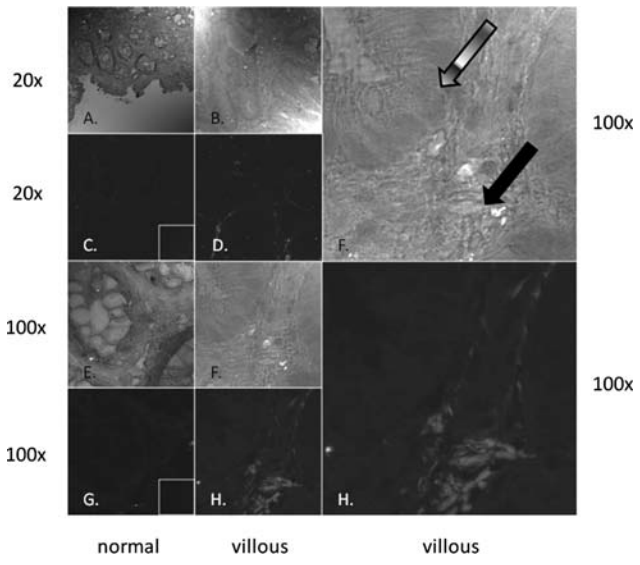

Figure 2. Confocal fluorescence microscopy for Myc immunohistochemistry in normal mucosa (panels A, C, E, G) and villous adenomas (panels B, D, F, H). Light microscopy (A, B, E, F) and fluorescence microscopy (C, D, G, H) are depicted at x20 and x100 magnification. Panels F and H are enlarged to demonstrate Myc expression in stromal components of the adenoma (solid black arrow) rather than mucosal components (grey arrow).

modulating the Wnt pathway, distinct from and in addition to *apc* mutation, are important in adenoma progression.

Several genes correlated with strong canonical (ß-catenin dependent) Wnt signaling have increased expression in villous adenomas. These include *wnt1*, *fz2*, *csnk2A2*, *pygo2*, *pygo1*, *frat2* and *myc*. Wnt1 is a secreted ligand that promotes canonical signaling (8) and frizzled 2 is one member of a family of cell surface receptors mediating canonical Wnt signaling (9). *csnk2A2* encodes casein kinase 2A2 which phosphorylates LEF1 to increase/stabilize ß-catenin binding (10) and enhance canonical Wnt throughput. Other studies have shown (11) that casein kinase II phosphorylates ß-catenin and directly augments its activity. The A2 subunit, also known as α-prime, can also bind to the cell cycle inhibitor p27 and inhibit its activity through phosphorylation. A large increase in *csnk2A2* in villous adenomas might inhibit p27 and prevent p27-mediated cell cycle inhibition. Pygo2 enhances canonical Wnt signaling in ways that are not fully understood (12). It is known to bind to methylated histones and probably stabilizes a ß-catenin/LEF/TCF complex, discouraging the formation of Groucho/LEF/TCF repressor complexes. Pygo2 is highly expressed in breast carcinoma cells and is required for their growth in tissue culture and in anchorage-independent assays (13). The role of Pygo1 in cancer is not well defined, though it appears, like Pygo2, to be involved in modulating Wnt activity in various developmental processes (14). FRAT2 is a GSK-3ß binding protein which is also a positive regulator of Wnt signaling and which has been reported to be upregulated in tumor progression (15).

Increased expression of *myc*, a classical Wnt target gene (16) in villous adenomas is evidence that canonical Wnt signal throughput is enhanced. The lack of upregulation in tubular adenomas suggests that Wnt pathway activating *apc* mutations is not sufficient to drive *myc* expression and that additional stimulation of Wnt signaling, or activation of other molecular pathways is necessary. Restriction of Myc expression in the stroma suggests a dynamic interaction between the mucosa, where Wnt signaling may be increased due to APC truncation and stromal cells within the polyp microenvironment. Gene expression in the stromal compartment of breast cancer has recently been shown to correlate with prognosis (17). An analogous situation may be present in colonic adenomas. In a prior study, (18) *c-myc* mRNA was found to be overexpressed in two-thirds of polyps though the histology of polyps was not reported. Myc protein has also been reported to have increased expression previously in colon cancers and in polyps (19,20). Its regulation, consistent with our findings, appears to be complex as polyps and cancers exhibit different patterns of intracellular expression in patients with familial adenomatous polyposis who harbor truncating (activating) mutations in *apc* (21). Wang *et al* (22) found incongruence between ß-catenin expression and Myc expression in colon cancers, implying that while Wnt signaling may upregulate transcription, c-Myc protein expression may be further regulated by post-transcriptional and translational mechanisms. Our data suggests that the operative mechanisms regulating Myc expression are complex, distinct between tubular and villous adenomas, and involve crosstalk between adenoma mucosal and stromal elements.

The successive increases in *ctnnb1* (ß-catenin) from normal to tubular adenomas to villous adenomas is interesting in that Wnt regulation of ß-catenin is primarily post-translational (23). However, Li *et al* (24) have suggested that ß-catenin transcription may in part be autoregulated by ß-catenin/TCF downstream targets, so the increasing expression may reflect ongoing augmentation of Wnt signals. Transcriptional regulation of ß-catenin may thus be an additional mechanism contributing to amplified Wnt throughput.

Several genes exhibiting increased expression in villous adenomas are involved in non-canonical Wnt signaling. Nemo-like kinase (NLK) induces a switch from Wnt/GSK3ß signaling to the Wnt/planar cell polarity (PCP) pathway (25). Wnt1 actually stimulates NLK via the MOM-4/Tak1 kinase signaling pathway (26) and NLK subsequently inactivates LEF/TCF's via phosphorylation and ubiquitylation (27). This is a negative regulatory loop resulting in the downregulation of canonical Wnt signaling. CXXC4 is also a negative regulator of canonical Wnt signaling, conferring its activity by binding with and inhibiting disheveled (Dvl) in the Dvl-Axin complex. Activation of Rho and Rac GTPases occurs in one the Wnt-mediated non-canonical pathway (28) and increased expression of *rho-u* is found in villous adenomas. The diaphanous related formin proteins Daam1 and Daam2 interact with Rho GTPases in regulating cell morphogenesis and the dynamics of the actin filament system in Xenopus (29). Activation of Rho for convergent extension requires Daam1 or Daam2. It is possible that the elevated levels of expression of *rho-u*, *daam1*, *damm2*, and perhaps *cxxc4* and *nlk*, mediate the alterations in cell morphology apparent in the transition from tubular to villous adenomas via inhibition of canonical and activation of non-canonical signals.

Naked cuticle homologue 1 (*nkd1*) is a Wnt antagonist that is regulated in part by Notch signaling (30). *nkd1*, along with the closely related *nkd2*, inhibit both canonical and non-canonical Wnt signaling in zebrafish (31). Increased expression of *nkd1* has been reported previously in adenomas (135-fold) and colon cancer (7.4-fold), suggesting a contribution by ß-catenin independent Wnt signaling pathways in colonic adenoma progression (32). The degree of expression induction in villous adenomas that we observed was considerably more modest (4-fold) than in the prior report and no induction was apparent in tubular adenomas.

Overall, these data suggest that, in adenoma histologic progression, Wnt signaling regulation is dynamic, not static, providing additional evidence that modulation of Wnt signal throughput is important even in the setting of downstream activating mutations in *apc* (6). These data suggest that canonical Wnt signaling is enhanced by elevated expression of *csnk2A2* and *pygo1/2*, possibly driven by upstream events (*wnt1* and *fz2*). Increased *myc* expression is evidence of this. The expression of several genes involved in non-canonical signaling are also increased in villous adenomas, possibly driven by the Wnt/Fz extracellular signals. Non-canonical events may contribute to the changes in histologic morphology apparent in the transition from tubular to villous adenomas.

Finally, the findings we present here have potential clinical significance in that a Wnt signaling molecular profile may be utilized to differentiate between tubular and villous adenomas. Eventually, this may be useful in identifying which polyps need colonoscopic removal. Defining a molecular profile may also be helpful in predicting the malignant potential

for adenomas of uncertain histology and Myc IHC may serve as a simple predictive marker for distinguishing adenomas of low and high malignant potential. In addition, the differences in Wnt-related gene expression suggest that non-*apc* components of the Wnt signaling pathway are involved in histological progression and that upstream intercessions that modulate Wnt signaling block this progression. This intimates a novel cancer prevention strategy for patients with a history of benign tubular adenomas.

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