Cell Surface Fucosylation Does Not Affect Development of Colon Tumors in Mice with Germline Smad3 Mutation

Steven E. Domino David M. Karnak Elizabeth A. Hurd

Department of Obstetrics and Gynecology, Cellular and Molecular Biology Program,
University of Michigan Medical Center, Ann Arbor, Mich., USA

Key Words

Carbohydrates • Colon cancer • Fucosyltransferase • Glycosylation • Transgenic mouse • Transforming growth factor- β signaling • *Ulex europaeus* agglutinin-I • α (1,2)Fucose

Abstract

Background/Aims: Neoplasia-related alterations in cell surface α (1,2)fucosylated glycans have been reported in multiple tumors including colon, pancreas, endometrium, cervix, bladder, lung and choriocarcinoma. Spontaneous colorectal tumors from mice with a germline null mutation of transforming growth factor- β signaling gene *Smad3* (*Madh3*) were tested for α (1,2)fucosylated glycan expression. **Methods:** *Ulex europaeus* agglutinin-I (UEA-I) lectin staining, fucosyltransferase gene Northern blot analysis, and a cross of mutant mice with *Fut2* and *Smad3* germline mutations were performed. **Results:** Spontaneous colorectal tumors from *Smad3* (–/–) homozygous null mice were found to express α (1,2)fucosylated glycans in an abnormal pattern compared to adjacent nonneoplastic colon. Northern blot analysis of α (1,2)fucosyltransferase genes *Fut1* and *Fut2* revealed that *Fut2*, but not *Fut1*, steady-state mRNA levels were significantly increased in tumors relative to adjacent normal colonic mucosa. Mutant mice with a *Fut2*-inactivating germline mutation were crossed with *Smad3*-targeted mice. In *Smad3* (–/–)/*Fut2* (–/–) double knockout mice, UEA-I lectin

staining was eliminated from colon and colon tumors; however, the number and size of tumors present by 24 weeks of age did not vary regardless of the *Fut2* genotype. **Conclusions:** In this model of colorectal cancer, cell surface α (1,2)fucosylation does not affect development of colon tumors.

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Introduction

Fucosylated oligosaccharides have been implicated in multiple cell-cell interactions in differentiation, development and malignancy [1, 2]. Glycosylated cell surface markers are used in cancer diagnosis, and therapeutic approaches have been designed to attack cancer cells via their glycans [3]. While previous studies have identified several α (1,2)fucosylated oligosaccharides associated with cancer progression, definitive evidence of a crucial role for these cell surface epitopes in carcinogenesis is lacking.

In human colon adenocarcinoma, expression of the fucosyltransferase gene *FUT1* has been found to correlate with malignant progression [4]. Steady-state mRNA levels of *FUT1* were elevated approximately 40-fold in all human colon adenocarcinomas tested and up to 340-fold in tumor-derived cell lines, while variable increases in mRNA levels of up to 50% were seen in adenomatous pol-

yps [4]. Transfection of a weakly tumorigenic rat colon carcinoma cell line with human FUT1 increased expression of $\alpha(1,2)$ fucose on CD44 polypeptide sequences encoded by exon V6 and concomitantly increased tumorigenicity of the cells in syngeneic rats and nude mice [5]. When a rat colon carcinoma cell line was transfected with antisense vectors for rat Fut1 and Fut2, opposite effects were found on tumorigenicity [6, 7]. Specifically, cells transfected with an antisense vector to rat Fut2 displayed decreased cell surface $\alpha(1,2)$ fucose on CD44 and decreased tumorigenicity. Cells transfected with an antisense vector to Fut1 were significantly more tumorigenic in syngeneic animals, but not in immunodeficient SCID mice. A potential counterreceptor for the $\alpha(1,2)$ fucose H antigen has been detected in rat colon cancer cells [8]. These data suggest a potential role for Fut1 and/or Fut2 and their cognate $\alpha(1,2)$ fucosylated products in malignant progression of several tumor types from rodents and humans.

Spontaneous colorectal tumors develop in mice carrying germline-inactivating mutations in the transforming growth factor- β (TGF- β) signaling gene *Smad3* (also called *Madh3*) [9]. It is not known whether these tumors express $\alpha(1,2)$ fucosylated glycans or fucosyltransferase genes. The data presented here examined colorectal tumors from these mice for *Ulex europaeus* agglutinin-I (UEA-I) binding, $\alpha(1,2)$ fucosyltransferase gene expression, and tested the importance of cell surface $\alpha(1,2)$ fucose in the progression of colon cancer by determining the extent of spontaneous colorectal cancer in *Smad3*-null ($-/-$) mice in the presence and absence of $\alpha(1,2)$ fucosylated oligosaccharides. This was accomplished by crossing Fut2-targeted mice with *Smad3*-targeted mice.

Materials and Methods

Animals

Animal protocols were approved by the University of Michigan Committee on Use and Care of Animals. *Smad3* (+/-) heterozygous mutant mice on the 129/T2SvEmsJ background were received from Parada and colleagues [9]. Mice were maintained under specific pathogen-free conditions, fed Purina chow 5001, and handled according to institutionally recommended guidelines. For the mutant mouse cross, the background of the Fut2 mouse on the 129X1/SvJ was estimated to be approximately 25% different compared to the 129/T2SvEmsJ background [10]. Therefore, to reduce the possibility of strain differences affecting tumor progression, Fut2 (+/-) heterozygous mutant mice (*Fut2^{tm1Sdo}* [11]) on the 129X1/SvJ background were backcrossed for three generations to wild-type 129/T2SvEmsJ mice (The Jackson Laboratory, Bar Harbor, Me., USA; stock No. 002065).

Lectin Staining

Prior to tissue collection, wild-type control and mutant mice were terminally perfused with ice-cold PBS followed by 4% paraformaldehyde. The entire colon was removed, cut longitudinally, and rinsed in ice-cold PBS. Grossly visible tumors were excised, weighed, and placed in formalin at 4°C for up to 1 week. Tissues were processed in paraffin blocks for permanent sections. Staining with UEA-I biotin-conjugated lectin (EY Laboratories, San Mateo, Calif., USA) was processed in a DAKO Autostainer Immunostaining System (Carpinteria, Calif., USA) by the University of Michigan IPOX histology core. Reagents used were peroxidase blocking reagent (S2001), labeled streptavidin-biotin (DAKO LSAB+ System), liquid DAB+ (K3468), and antibody diluent (S3022) at a 1:40 dilution.

Northern Blot Analysis

Paired distal colon tumors and adjacent control colon sample were excised and flash-frozen from *Smad3*-null mice at 24 weeks of age. Total RNA was isolated by the guanidine isothiocyanate/phenol method by homogenizing samples in 1 ml of TRIzol (Gibco BRL) per 100 mg of tissue following the manufacturer's protocol. Polyadenylated mRNA was then isolated by QIAGEN Oligotex midi mRNA columns. After estimating concentrations of the samples by absorbance at 260 nm, 2 μ g of mRNA was loaded per lane for Northern blot analysis. Blots were sequentially probed with [³²P]dCTP random-primed cDNA probes for Fut2 (214-bp probe [12]), control gene mouse cyclophilin-A (721-bp probe, Ambion mouse DECAprobe template 7375) and control mouse ribosomal protein L32 (113-bp probe purified from a *Hind*III digestion of Pharmingen mouse L32 housekeeping template 45181P) at a specific activity of 1–2 $\times 10^9$ cpm/ μ g of DNA probe. Duplicated blots were probed with the Fut1 probe, an antisense RNA probe was generated by T7 RNA polymerase with [³²P]UTP at a specific activity of $\sim 1 \times 10^{10}$ cpm/ μ g of probe to quantify the paucity of Fut1 mRNA (228 bp probe 'C' [13]), followed sequentially by probing for cyclophilin-A and ribosomal protein L32. After hybridization, all blots were washed under high stringency at 65°C with 0.2 $\times$ SSC and 0.2% SDS, then placed on a PhosphorImager screen for quantification (Molecular Dynamics).

Cyclophilin-A and ribosomal protein L32 were chosen for testing as potential internal standard controls since a search of NCI Cancer Genome Anatomy Project (CGAP) library browser showed no difference in expression of these two genes (cyclophilin-A: UniGene Hs.9880; L32: UniGene Hs.169793) in a human colon cancer cDNA library (NCI_CGAP_Co8) when compared to normal human colon (NCI_CGAP_Co3) by digital differential display. On experimental Northern blots, equivalent loading differences were seen with both control genes. Expression of L32 was chosen as an internal reference to standardize loading between samples. Relative expression in tumor versus control colon samples was compared by two-tailed t test ($n = 4$).

Mutant Mouse Cross

To generate double mutant mice, breeding pairs of *Smad3* (+/-) heterozygous mutant mice and Fut2 (+/-) heterozygous mutant mice were crossed (*Fut2* and *Smad3* map to separate mouse chromosomes; Chr 7 and Chr 2, respectively). Heterozygous *Smad3* (+/-) mice were chosen for the cross since *Smad3* ($-/-$) homozygous null mice did not breed well. Eight breeding cages were maintained for 6–8 months to generate sufficient progeny to

Fig. 1. UEA-I lectin staining of normal colonic mucosa and tumors of 24-week-old Smad3 homozygous null mice. The colons of Smad3 (-/-) null mice were prepared for UEA-I lectin immunohistology as described in Materials and Methods. Sections are oriented with the luminal surface at the top and submucosa below. **a** Non-neoplastic colon crypts with apical staining of goblet cells (bar = 20 μ m). **b** Higher magnification of goblet cell granules (bar = 20 μ m). **c** Abnormal crypts with UEA-I binding of apical surfaces and enclosed secretions (bar = 100 μ m). **d** Adjacent control section stained identically to **c** except without UEA-I lectin showing little nonspecific staining (bar = 100 μ m).

meet the number of mice estimated to be required in a prestudy power analysis. Genotyping was performed on all progeny by PCR using specific primers on tail tip DNA, and confirmed by a second DNA sample from ear tip biopsy in all Smad3 (-/-) null mice. Smad3 (+/+) wild-type and Smad3 (+/-) heterozygous progeny were euthanized. Smad3 (-/-) null mice were then genotyped for Fut2 and housed until 24 weeks of age. Four animals that showed signs of distress (ruffled fur, rectal prolapse from tumor) were euthanized and tissues collected at 22–23 weeks of age, otherwise all mice were euthanized and tissues collected at 24 weeks.

Results

UEA-1 Lectin Staining and $\alpha(1,2)$ Fucosyltransferase Gene Expression Differ between Normal Colonic Mucosa and Neoplastic Mucosa

Colon tumors of 24-week-old Smad3 (-/-) null mice were examined for expression of $\alpha(1,2)$ fucosylated epitopes by UEA-I lectin binding. Lectin staining in colonic tumors was associated with abnormal crypt secretions

and cell surfaces. Adjacent nonneoplastic mucosa showed positive UEA-I binding concentrated in goblet cell granules (fig. 1). The locations of UEA-I reactivity in Smad3 null mice were similar to differences in UEA-I binding between human adenocarcinomas and normal colonic mucosa [14]. We previously have shown that goblet cell granules are the predominant site of UEA-I reactivity in colon correlating with production of $\alpha(1,2)$ fucosylated mucin Muc2 secreted from goblet cells [15].

UEA-I binding is closely associated with both Fut1 and Fut2 $\alpha(1,2)$ fucosyltransferase gene expression in secretory tissues including the colon, stomach, and uterus [11]. To determine which $\alpha(1,2)$ fucosyltransferase gene(s) were expressed in tumors and the relative differences in steady-state mRNA levels between tumors and normal colon, Northern blot analysis was performed. Polyadenylated RNA samples were isolated from spontaneous colon tumors and adjacent normal colon from four Smad3 homozygous null mice as described under Experimental Methods. Gene-specific probes were utilized for three mouse

Fig. 2. Expression of Fut1 and Fut2 in distal colon tumors of 5- to 6-month-old Smad3 homozygous null mice. Polyadenylated RNA was isolated from spontaneous colon tumors and normal colon from four Smad3 homozygous null mice. Two micrograms of polyadenylated RNA per lane were electrophoresed by formaldehyde agarose gel electrophoresis, transferred to nylon membranes, hybridized with gene-specific probes, and quantified by PhosphorImager. The columns display the mean $\pm$ the standard deviation in relative mRNA expression between tumor and control colon samples. Statistically significant elevation of Fut2 steady-state mRNA levels was found in tumors compared to adjacent normal colon (* $p < 0.05$). Sample autoradiographs for L32 control, Fut1 and Fut2 gene probes are shown below.

$\alpha(1,2)$ fucosyltransferase genes, *Fut1*, *Fut2*, and *Sec1*. Fut1 steady-state mRNA levels did not differ between control colon and tumors while Fut2 steady-state mRNA levels were increased in colon tumors (fig. 2). Sec1 was not detected in normal colon or tumors (data not shown).

Cross of Mice with Germline Mutations in Fut2 and Smad3 Produced Tumors That Lack UEA-I Binding but Do Not Vary in Number or Size Based on Fut2 Background

To determine the importance of elevated Fut2 expression in Smad3 $-/-$ tumors, Fut2-targeted mice were crossed with Smad3-targeted mice. For generation of double mutant mice, breeding pairs of Smad3 (+/-) heterozygous mutant mice and Fut2 (+/-) heterozygous mutant mice were crossed. Smad3 $-/-$ null progeny were genotyped for Fut2 and categorized into Fut2 (+/+) wild-type control, Fut2 (+/-) heterozygous, and Fut2 $-/-$ homozygous null. Tumor burden of Fut2 (+/-) Smad3 $-/-$ mice (experimental group 1) was predicted to be similar to Fut2 (+/+) Smad3 $-/-$ littermates (positive control group). Double null Fut2 $-/-$ Smad3 $-/-$ mice were predicted to have decreased tumor burden (experimental group 2). To estimate the sample size needed for the number of mice required for the cross, the following assumptions were made: (1) average number of colon tumors per mouse of 3.8 with a range of 2–6 gross tumors and standard deviation of 1.15 [9], (2) amount of change in tumor burden deemed to be clinically significant: 50%, and (3) an effect size of 1.65 (1.9 divided by standard deviation of 1.15). To detect with statistical significance a 50% increase or decrease in tumor burden with a power of 0.9

Fig. 3. UEA-I lectin staining of colon tumors of 24-week-old Smad3 $-/-$ /Fut2 $-/-$ double homozygous null mice. Colon tumors from mice with germline mutations of Smad3 and Fut2 were tested for UEA-I lectin staining. **a** Tumors Fut2 (+/-)/Smad3 $-/-$ stained extensively with UEA-I lectin similar to tumors from Smad3 $-/-$ mice with wild-type Fut2 (+/+) expression (fig. 1). **b** Tumors from double null Fut2 $-/-$ /Smad3 $-/-$ mice lack specific UEA-I staining indicating expression of Fut2 is required to generate $\alpha(1,2)$ fucosylated epitopes (bar = 100 μ m).

Table 1. Cross of mice with germline mutations of Smad3 and Fut2 comparing tumor number and size

Genotype:	Control group Fut2 (+/+)/Smad3 (-/-)	Experimental group 1 Fut2 (+/-)/Smad3 (-/-)	Experimental group 2 Fut2 (-/-)/Smad3 (-/-)
Number of progeny	12	21	10
Number of mice with tumors	5	12	5
Number of colon tumors per affected mouse	1.4 ± 0.5	1.3 ± 0.5	1.2 ± 0.5
Average weight of tumors, mg	156 ± 60	131 ± 58	142 ± 48

and $p < 0.05$, 10 animals were estimated to be required in each group.

Eight breeding cages were maintained for 6–8 months to generate sufficient progeny to meet the sample size estimated. The distribution of progeny obtained fit the predicted distribution for a Fut2 (+/-) heterozygous cross of approximately 25% wild type, 50% heterozygous, and 25% null suggesting no embryonic lethal effects from loss of the Fut2 gene on the Smad3 null background. Tumors were obtained in all three genotypes of mice, confirming the original observation by Zhu et al. [9] that Smad3 (-/-) mice develop spontaneous colorectal tumors, albeit at a lower penetrance (~50%) in the environment for this study.

In Smad3 (-/-)/Fut2 (-/-) double knockout mice, UEA-I lectin staining was eliminated from colon and colon tumors (fig. 3), indicating significant loss of $\alpha(1,2)$ fucosyltransferase function, although the Fut1 gene was not targeted in this experiment and a trace amount of $\alpha(1,2)$ fucosylated glycans may be present below the level of detection by UEA-I lectin. The number and size of tumors present by 24 weeks of age did not vary regardless of the Fut2 genotype (table 1). These data suggest in this genetic model of colorectal cancer that cell surface fucosylation does not affect development of colon tumors.

Discussion

Aberrant expression of fucosylated glycans has been detected in tumors from multiple tissues including pancreas [16], liver [17], endometrium [18], and colon [19, 20]. In at least some tumors, the mechanism appears to be an increased expression of glycosyltransferase enzymes [21–23]. To test the importance of glycosyltransferase expression in tumors, previous investigators have crossed mutant mice to observe the resulting effect on

tumor development. With this approach, the relevance of N-acetylglucosaminyl epitopes in breast cancer metastasis was shown by crossing $\beta(1,6)$ N-acetylglucosaminyl transferase V null mice with transgenic mice that develop metastatic mammary tumors [24, 25]. Specific to human colorectal cancer, metastatic potential was shown to dramatically decrease in cultured cells deficient in the GDP-fucose-generating enzyme FX, potentially by altered interaction of fucosylated glycans on colorectal cancer cells with the extracellular matrix [26].

Additional approaches to the study of colon tumors have included transfection of carcinoma cell lines with human fucosyltransferase genes to increase cell surface elaboration of $\alpha(1,2)$ fucose. Le Pendu and colleagues [5] found increased fucosylation of CD44 polypeptide sequences encoded by exon 6 and concomitantly increased tumorigenicity of the cells in syngeneic rats and nude mice upon transfection with human Fut1. Alteration of CD44v6 fucosylation has been suggested to mediate apoptosis of colon cancer cells since apoptosis induction was associated with decreasing $\alpha(1,2)$ fucosylated blood group antigen expression and fucosyltransferase activity [27]. In particular, all $\alpha(1,2)$ fucose-containing antigens (blood groups A, H and Lewis groups) showed a loss of expression, predominantly from CD44 glycoprotein. In a comparison of cell lines with differing extent of $\alpha(1,2)$ fucosylation and tumorigenicity, high expression of $\alpha(1,2)$ fucose was correlated with a decreased sensitivity to apoptosis [27]. Since elimination of mature cells in the gastrointestinal epithelium is likely mediated by apoptosis, impairment of apoptosis has been proposed to occur in close association with initiation of colon carcinogenesis [28].

Our study sought to determine if increased Fut2 expression evidenced in Smad3 knockout mice had an effect on tumor number, progression, or metastatic potency. By crossing Fut2 null mice with Smad3 nulls, we effectively eliminated $\alpha(1,2)$ fucosylation in the colon. If

such fucosylation was an important factor in tumor progression, one would expect fewer or smaller tumors. Our results found no significant difference in tumor size or number between Smad3 nulls and Smad3/Fut2 double nulls. Unfortunately, our model system could not address effects on metastatic potential because mice sacrificed at 24 weeks for humane purposes had tumors localized to the mucosa. In our hands, Smad3 null mice displayed a tumor penetrance lower than originally reported. It should be noted that three lines of Smad3 null mice have been generated and only one of them, on the 129/T2SvEmsJ background, develops colorectal tumors, therefore strain background has a significant effect on tumor formation [29, 30].

The potential mechanism for fucosyltransferase interaction with tumorigenicity is complicated by the variety of substrates and differing substrate specificity for the fucosyltransferase enzymes [31]. Fut1 and Fut2 enzymes can $\alpha(1,2)$ fucosylate a variety of glycans and glycolipids. In enzymic studies with natural and synthetic substrates, both enzymes were shown to be capable of adding $\alpha(1,2)$ fucose to both H antigen type 1 and type 2 acceptor substrates but with differing enzymatic kinetic parameters [13, 32]. In pancreatic cancer cells, studies have postulated that increased $\alpha(1,2)$ fucosylation reduce metastatic potential by decreasing the adhesive properties due to altered sialyl Lewis antigen presented at the cell surface [33].

The data in the present study may also be influenced by effects on the immune system. Alteration of TGF- β

signaling in mice with germline mutation of Smad3 is not restricted to the colonic epithelium. In T cells, loss of TGF- β signaling leads to abnormal differentiation and activation compromising mucosal immunity [30]. Goupille et al. [34] reported an effect of $\alpha(1,2)$ fucosylation on the in vivo growth of colon carcinoma cells in an experimental rat model. The effect was visible in immunocompetent animals only, whereas $\alpha(1,2)$ fucosyltransferase transfected cells and control cells grew similarly in immunocompromised animals. Likewise, an effect of $\alpha(1,2)$ fucosylation on immune escape may not be visible in Smad3 $-/-$ mice.

The possibility exists that Fut2 overexpression in Smad3 nulls is an unimportant consequence of a tumor-related cellular change. However, the converse could also be true, i.e. Fut2 overexpression could be a compensatory change that decreases metastatic potency. In a future study, this question could be addressed by injecting tumor-derived cells from the experimental groups studied here into nude mice and analyzing tumor occurrence.

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