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Increased colon tumor susceptibility in azoxymethane treated CEABAC transgenic mice

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Human carcinoembryonic antigen (*CEA*), a widely used clinical tumor marker, and its close relative, *CEACAM6*, are often overexpressed in many cancers. This correlation suggests a possible instrumental role in tumorigenesis, which is supported by extensive results obtained with several *in vitro* systems. The implication that these results could also apply *in vivo* warrants investigation. Since mice do not possess homologs of the glycophosphatidyl inositol (GPI)-anchored CEACAM family genes *CEA*, *CEACAM6* and *CEACAM7*, we have constructed transgenic mice harboring a 187 kb portion of the human CEACAM family gene locus contained in a bacterial artificial chromosome (CEABAC) that includes genes coding for *CEA*, *CEACAM6* and *CEACAM7*. In this study, we treated the CEABAC mice and their wild-type littermates with azoxymethane (AOM) in order to induce colon tumor formation. At 20 weeks post-treatment, the CEABAC transgenics showed more than a 2-fold increase in mean tumor load relative to their wild-type littermates. Cell surface expression of *CEA* and *CEACAM6* increased by 2- and 20-fold, respectively, in colonocytes from the tumors relative to colonocytes from non-AOM treated transgenics and a de-regulated spatial pattern of *CEA/CEACAM6* expression was found in 'normal' crypts adjacent to the tumors, thus mimicking closely the situation in human colon tumorigenesis. A modestly increased incidence of β-*catenin* mutations also observed in the AOM-induced CEABAC tumors. These results show that expression of the human GPI-anchored CEACAM family genes predisposes mice to acquire and/or retain essential mutations necessary for sporadic colon tumor development.

Introduction

Human carcinoembryonic antigen (CEA) family genes, that encode a group of highly glycosylated homotypic/heterotypic intercellular adhesion molecules, are closely clustered on chromosome 19q13.2 and represent a subset of the immunoglobulin superfamily (1). The glycophosphatidyl inositol (GPI)-anchored family members, *CEA* and *CEACAM6*, are overexpressed in as many as 70% of all human tumors, a fact that underlies their widespread use as tumor markers in the cancer clinic (2–4). This overwhelming correlation suggests an instrumental role for these molecules in tumorigenesis. In human patients, overexpression of *CEA* and *CEACAM6* is often associated with poor prognosis (2,4). Overexpression of *CEA* and *CEACAM6 in vitro* was also shown to elicit various tumorigenic effects, i.e. blockage of cell differentiation (5–7), inhibition of anoikis/apoptosis (8–10) and disruption of tissue architecture (7). More direct evidence utilizing *in vivo* models, however, has been difficult to acquire due to the lack of informative animal models, since GPI-anchored CEACAM family genes are present only in primates (1,11,12). *CEA*-only transgenic mice were previously constructed to test this hypothesis but showed no obvious tumorigenic phenotype (13,14) nor synergistic effects by mating with other tumor-prone mouse models, including those bearing *APC* mutations (15). This could be due to expression levels that were below a threshold level required for tumorigenesis or to the absence of other human GPI-anchored CEACAM family members, notably *CEACAM6*.

A mouse model that better mimics the human situation, in which part of the human CEACAM family gene locus, including genes coding for *CEA*, *CEACAM6*, *CEACAM7* and *CEACAM3*, propagated in a 187 kb BAC (CEABAC), has been constructed recently on the FVB genetic background (16). Two independent transgenic lines showed remarkably similar expression patterns of these CEACAM family genes to that of humans (16). The GPI-anchored CEACAM family genes *CEA*, *CEACAM6* and *CEACAM7*, but not the transmembrane-anchored gene *CEACAM3*, are expressed in the transgenic colonocytes (16). The colonic expression of the three GPI-anchored genes, at least at the levels seen in these two CEABAC transgenics, however, does not lead to colon tumor formation (16).

1,2-Dimethylhydrazine (DMH) and its active metabolite, azoxymethane (AOM), are colon-specific carcinogens that induce focal colon tumors in susceptible murine strains. The guanine bases in genomic DNA can be methylated by AOM forming O^6-methylguanine adducts. The latter can either be repaired by methylguanine methyltransferase (MGMT) (17) or trigger apoptosis (18). Actively proliferating cells that escape these two protective mechanisms will acquire G→A base conversions after two rounds of DNA replication (19). Mutations that provide survival or growth advantage will be selected for and will be present in tumors. For example, mutations in β-*catenin* are frequently found in AOM-induced colon tumors (20); those in *Kras* and *p53* occur but are less common (20–22). Hence, this carcinogen provides a useful tool to assess colon tumor susceptibility in different murine strains, including transgenics and knockouts, whose cells may have different rates of apoptosis, proliferation and DNA repair.

To evaluate the tumorigenic effects of *CEA* and *CEACAM6* anticipated by the *in vitro* studies, the CEABAC mice were given weekly injections of AOM for 6 weeks. A highly

Abbreviations: AOM, azoxymethane; BAC, bacterial artificial chromosome; CEA, carcinoembryonic antigen; CEACAM, CEA family cell adhesion molecule; CEABAC, BAC bearing part of the human CEACAM family gene locus; DMH, 1,2-dimethylhydrazine; GPI, glycophosphatidyl inositol; GSK-3β, glycogen synthase kinase-3β; MGMT, methylguanine methyltransferase; PCR, polymerase chain reaction; WT, wild-type.

significant 2-fold increase in tumor load was obtained in the CEABAC mice at 20 weeks post-treatment. A de-regulated cryptal expression pattern of *CEA*/*CEACAM6* and a 2- and 20-fold overexpression of *CEA* and *CEACAM6*, respectively, were observed in these tumors, exactly as seen in human colon cancers. A marked increase in the incidence of β-*catenin* mutations per mouse was also observed in the AOM-treated CEABAC mice although this increase per tumor was modest. Thus expression of the human GPI-anchored CEACAM family genes normally found in human colonocytes predisposes mice to acquire and/or retain essential mutations necessary for sporadic colon tumor development.

Materials and methods

Animals

The generation of the CEABAC transgenic mice harboring a 187 kb BAC that contains complete human *CEA*, *CEACAM6*, *CEACAM7* and *CEACAM3* genes was reported elsewhere (16). These mice were generated and maintained on the FVB genetic background. The CEABAC2 and CEABAC10 transgenic lines possess 2 and 10 copies of the transgenes, respectively, in a head to tail orientation in a single cluster. Age-matched wild-type (WT) controls were obtained from breeding between CEABAC and FVB mice (Harlan Bioproducts for Science, Indianapolis, IN). All mice were housed under controlled conditions of a 12 h light/dark cycle, 23 ± 2°C room temperature and 50 ± 10% relative humidity. Food and water were available *ad libitum*.

AOM treatment and analytical methods

For tumor induction, 3–4 month-old CEABAC2, CEABAC10 and WT mice were treated with 6 weekly intra-peritoneal injections of AOM (obtained from the National Cancer Institute, Bethesda, MD) at a dose of 10 mg/kg body wt. Untreated 3–4 month-old CEABAC2, CEABAC10 and WT mice were the negative controls for the experiment. Animals were sacrificed at 20 weeks after the last injection. Colons were cut open longitudinally, fixed with 4% paraformaldehyde for 16 h at 4°C, stained with 2% methylene blue and examined under a dissecting microscope. Tumor number and volume (length × width × height) were recorded for each AOM-treated mouse.

Immunohistochemical analysis

For immunohistochemical analysis, tissues were fixed with 4% paraformaldehyde for 16 h at 4°C and quickly frozen with isopentane at −70°C. Frozen sections, of 7 μm thickness, were obtained using a cryostat at −25°C. Frozen sections were stained for human CEACAM family members using rabbit polyclonal anti-human CEA antibody at a dilution of 1:10 000 and anti-rabbit Envision Reagent (DAKO Diagnostics Canada, Mississauga, ON). Sections were developed with DAB (3′3′-diaminobenzidine) for 5 min and were counterstained with Mayer's hematoxylin (Sigma-Aldrich Canada, Oakville, ON).

Fluorescence-activated cell sorting (FACS) analysis

Colon tumor, normal colonic and normal spleen tissues were obtained from freshly resected AOM-induced tumors, untreated normal mucosa and spleen, respectively. Tissues were cut into fine pieces and treated with collagenase solution as described previously (23). Single cell suspensions were fixed with 2% paraformaldehyde for 16 h at 4°C. Cells were labeled with mouse monoclonal antibodies, A20 (detecting *CEA* but not *CEACAM6*) or 9A6 (detecting *CEACAM6* but not *CEA*) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies. Labeled cells were analyzed by FACScan® (Becton Dickinson, Bedford, MA).

Mutational analysis

Frozen tumors were digested with Proteinase K solution (500 ug/ml Proteinase K, 50 mM Tris, (pH 8.0), 100 mM EDTA, 0.05 mM $CaCl_2$ and 0.5% SDS) at 55°C for 16 h. Genomic DNA was then purified using a standard phenol–choroform extraction method (24). Exon 3 of the β-*catenin* gene, exon 1 of the *Kras* gene, and exons 5–6 and 7–8 of the *p53* gene were amplified by PCR using *Pfu* Turbo® (Stratagene, La Jolla, CA) and specific primers (β-*catenin* exon 3: 5′-GCTGACCTGATGGAGTTGGA-3′ and 5′-GCTACTTGCTCTTGCGTGAA-3′, product size = 227 bp; *Kras* exon 1: 5′-TGAGAGCCATTAGCTGCTAC-3′ and 5′-CCTCTATCGTAGGGTCGTAC-3′, product size = 392 bp; *p53* exons 5-6: 5′-CGTCCAATGGTGCTTGGACAA-3′ and 5′-AAGGTACCACCACGCTGTGGC-3′, product size = 462 bp; *p53* exons 7-8: 5′-GCCGGCTCTGAGTATACCACC-3′ and 5′-CGCCTGCGTACCTCTCTTTGC-3′, product size = 577 bp). PCR products were sequenced using both forward and reverse primers with an automated DNA sequencer. Mutations were detected by observing individual chromatograms (see Figure 4).

Statistical analysis

Mean tumor loads and mean tumor volumes of different animal groups were compared using the unpaired two-tailed Student's *t*-tests. Frequencies of specific gene mutations in different animal groups were compared using the two-tailed Fisher's exact tests. The results were considered statistically significant if the *P*-values were <0.05.

Results

Human CEACAM family gene expression increases AOM-induced tumor incidence

To assess the effect of GPI-anchored CEACAM family members on colon tumorigenesis, WT and CEABAC transgenic mice were given 6 weekly intra-peritoneal injections of AOM. FVB mice, the strain used in this study, do not normally develop spontaneous colon tumors but are susceptible to AOM-induced colon tumor formation (25). Fifty-two WT and sixty CEABAC mice were treated with AOM and thirty-eight WT and fifty-one CEABAC mice were left untreated as controls. As expected, the untreated WT and CEABAC mice showed no evidence of colon tumors. Many of the AOM-treated mice, however, had bloody stools or diarrhea and some had prolapse of the rectum. At 20 weeks post-treatment, while the AOM-treated WT mice bore on average 6.2 ± 0.2 tumors/colon, the AOM-treated CEABAC mice bore 14.4 ± 0.3 tumors/colon (Table I), which is a highly significant difference ($P < 0.0001$). This difference in incidence is most apparent in a plot of single animal tumor burden, demonstrating that most of the AOM-treated WT mice had <6 tumors/colon, whereas most of the AOM-treated CEABAC mice had >12 tumors/colon with some bearing as many as 25 tumors/colon (Figure 1).

All AOM-induced tumors were present in the distal colon and rectum, as previously reported (25). There was no apparent difference in the tumor load between male and female animals ($P > 0.05$); no correlation between body weight and tumor load ($R^2 < 0.95$) could be shown (data not shown). The mean tumor volumes for the WT versus CEABAC mice showed no significant difference (Table I). Histologically, the tumors were adenomas and adenocarcinomas in both WT and CEABAC mice. Preliminary data showed no significant difference in the proportion of adenomas and adenocarcinomas between WT and CEABAC mice (data not shown). The increase in incidence was observed for both CEABAC2 and CEABAC10 mice, two independent transgenic lines, ruling out possible effects of the integration site of the transgenes. Thus, the expression of human GPI-anchored CEACAM family genes in mouse colon increases tumor formation induced by carcinogen treatment.

Expression of CEA and CEACAM6 is de-regulated after AOM treatment

In human colon tumors, *CEA* and *CEACAM6* are often overexpressed (2,4), whereas expression of *CEACAM7* is usually absent (26). To determine whether this important feature of human tumors was also present in the AOM-induced tumors of CEABAC transgenic mice, single colonocyte suspensions of the colorectal tumors and control tissues (normal colon epithelium and spleen) were obtained from both WT and CEABAC mice. Cell surface expression levels of *CEA* and CEACAM6 were determined by FACS analysis using specific

Table I. Tumor load in AOM-treated animals at 20 weeks post-treatment

Mouse strain[a]	Number of animals	Tumors/colon[b] (Mean ± SEM)	Tumors/colon[b] Range	Mean tumor volume[b] (mm^c ± SEM)
WT	19	6.2 ± 0.8	1–14	16.6 ± 3.1
CEABAC2	13	13.9 ± 1.6[d]	2–22	
CEABAC10	12	15.0 ± 2.2[d]	4–25	
CEABAC total[c]	25	14.4 ± 1.3[d]	2–25	15.6 ± 1.9[e]

[a]Mice were treated with six weekly i.p. injections of 10 mg/kg AOM and sacrificed at 20 weeks after the last injection. No lesions were found in untreated mice. A low proportion (6%) of the treated mice (7/112) were found dead during the 6 week injection period, probably due to acute drug toxicity. Thirty-four mice were sacrificed at earlier time points (6 and 12 weeks post-treatment) for early assessment. At 20 weeks post-treatment, 82% of the AOM-treated mice (64/78) survived relative to 100% for the untreated animals.
[b]Scored for tumors sized $\geq$ 1 mm^3; SEM = standard error of the mean.
[c]CEABAC2 + CEABAC10.
[d]$P < 0.0001$, compared with WT.
[e]$P > 0.05$, compared with WT.

monoclonal antibodies. Cell surface expression levels of *CEACAM7*, however, could not be detected in normal and tumor samples using this method due to its low expression levels (data not shown). Since *CEACAM6* is expressed in neutrophils (16) that are present in the normal colonic epithelia and colon tumors, splenocytes (which include neutrophils) were obtained and subjected to FACS analysis in order to allow removal of any such contribution from the colonocyte FACS profiles (Figures 2A–C). As expected, all single cell suspensions from WT tissues were negative for *CEA* and *CEACAM6*, i.e. similar to background levels (data not shown). In the AOM-induced tumors from the CEABAC mice, mean cell surface expression levels of *CEA* and *CEACAM6* in the tumor colonocytes were 2- and 20-fold higher, respectively, than the levels in normal colonocytes from untreated CEABAC mice (Figure 2D and E). It is of interest that these cell surface expression ratios were observed to be similarly higher for *CEACAM6* than for *CEA* in purified colonocytes from human colorectal carcinomas (23).

In normal human colonic epithelium, *CEA* and *CEACAM6* are mostly expressed in fully differentiated cells near the mucosal surface and on the apical surface of the cells (23), which was also observed in the CEABAC mice (Figure 3A). This cryptal expression pattern of *CEA* and *CEACAM6* is usually lost, i.e. similar expression levels are observed throughout the crypts, not only in tumors but also prior to and during tumor formation in 'normal' human epithelium adjacent to the tumor (23). Moreover, in the adjacent 'normal' human epithelium, the expression of *CEA* and *CEACAM6* is no longer restricted to the apical surface of colonocytes, but is seen on the basolateral surface as well and, in some cases, in the cytoplasm (23). This de-regulated expression pattern, known as the 'field effect' (27), was also observed in the AOM-treated CEABAC mice (Figure 3C).

Incidence of mutations in β-catenin, Kras and p53 increases in AOM-treated CEABAC mice

All tumors of size >1 mm^3 were collected from three WT and three CEABAC mice 20 weeks after completion of the AOM treatment; 19 and 33 tumors were obtained respectively.

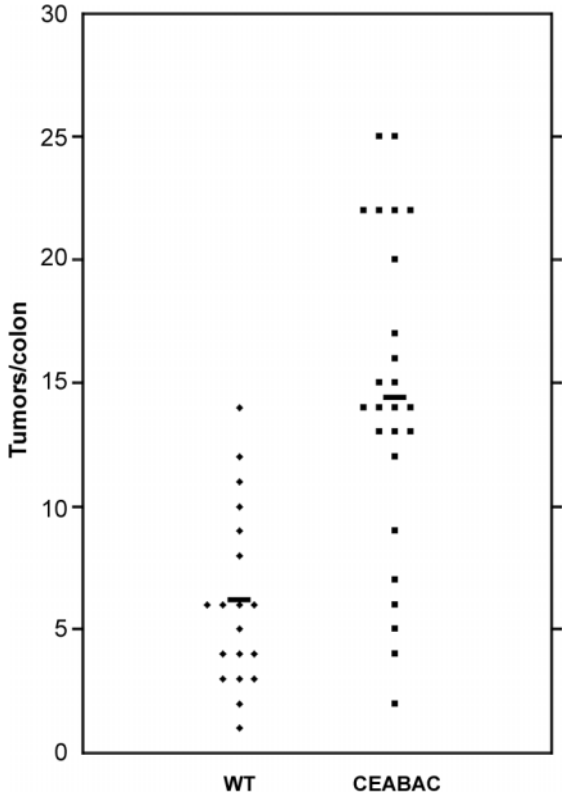

Fig. 1. Tumor load of individual animals. Each dot represents the number of colon tumors (with tumor volume > 1 mm^3) in a single animal (WT, closed diamonds; CEABAC2 or CEABAC10, closed squares) at 20 weeks post-treatment of AOM. The horizontal bar represents the average tumor load for each group of animals (WT, 6.2 tumors/colon; CEABAC, 14.4 tumors/colon). Note that most WT mice had <6 tumors/colon and most CEABAC mice had >12 tumors/colon.

Tumor DNA was subjected to sequence analysis of the mutational hotspots on the β-*catenin, Kras* and *p53* genes. Mutations in exon 3 of the β-*catenin* gene that contains the glycogen synthase kinase (GSK)-3β-phosphorylation consensus motif are frequently found at codons 32, 33, 34, and 41 in AOM-induced rodent colon tumors (28). Similar mutations were obtained here (Table II) with the sequencing evidence shown in Figure 4. An increase in the percent of tumors with *β-catenin* mutations in the CEABAC (64%) versus WT mice (53%) and a shift of mutation frequency from codon 41 to codons 32/34 in the CEABAC tumors (10 and 80%, respectively) versus WT tumors (30 and 60%, respectively) were observed (Table II). Including the fact that there were about twice as many tumors per mouse in the AOM-treated CEABAC mice, the incidence of β-*catenin* mutations was about twice that of the WT mice (7.0 versus 3.3).

Mutations in the *Kras* gene can be commonly found at codon 12 or 13 in both human cancers (29) and AOM-induced tumors (28). One G12D and one G13D mutation were found in the CEABAC tumors (6% of all tumors), whereas one G12S mutation was found in the WT tumors (5% of all tumors) (Table II and Figure 4). It was shown previously that *Kras* mutations are present in 0–10% of AOM-induced mouse tumors and at much higher frequencies in AOM-induced rat tumors (28) and human tumors (29).

Mutations in the *p53* gene are scattered throughout exons 5–8 with higher frequency at codons 175, 248 and 273 in human colon cancers (30). The mutation frequency is much

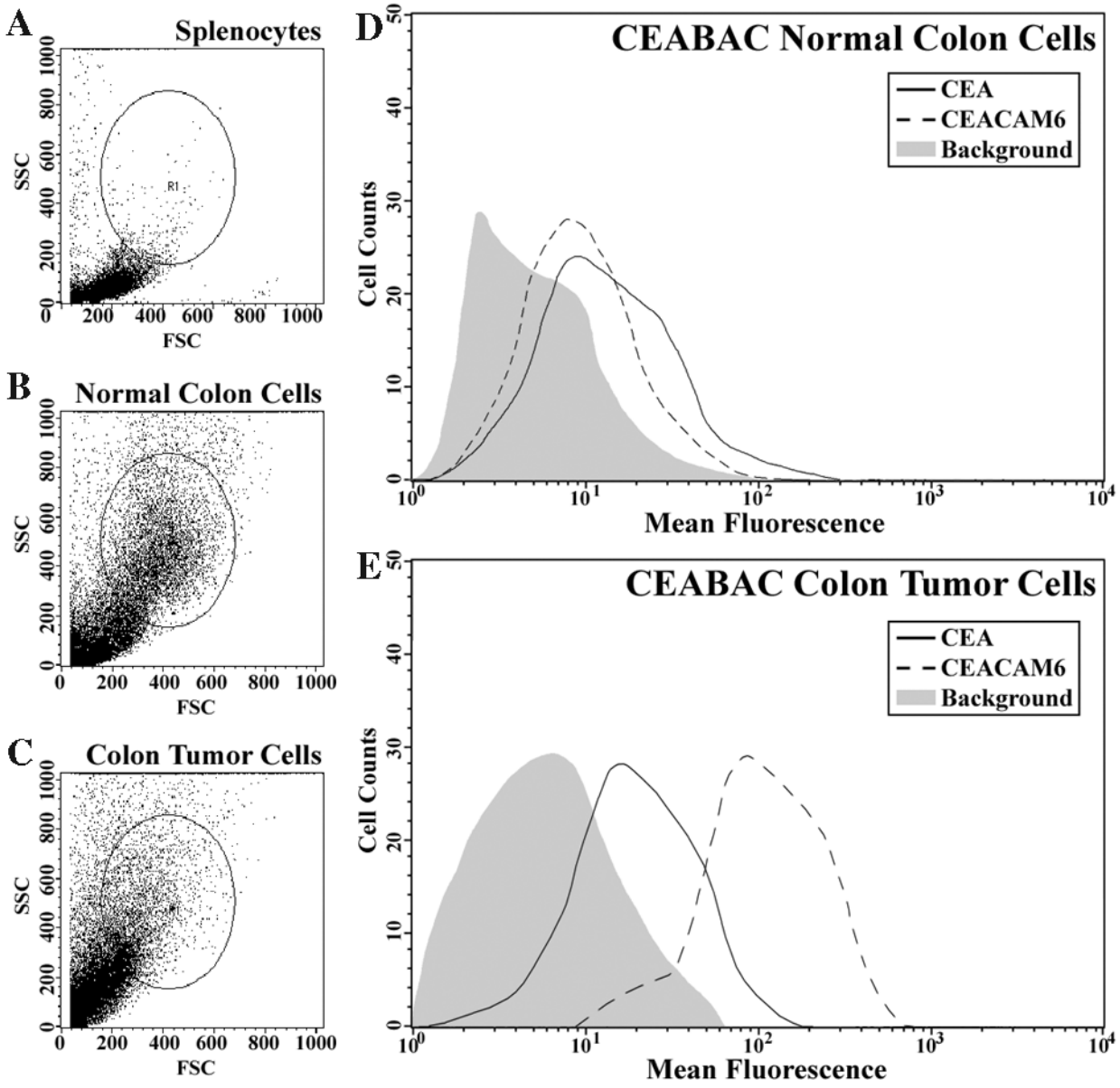

Fig. 2. Cell surface expression of *CEA* and *CEACAM6*. Dot plots relating side scatter (SSC), i.e. inner complexity of the cell (shape of the nucleus, degree of cytoplasmic granulosity or membrane roughness), to forward scatter (FSC), i.e. cell volume, of (**A**) splenocytes, (**B**) normal colon cells, and (**C**) colon tumor cells. The circles represent the cell populations (R1) gated for analysis, which were set to exclude hematopoietic cells that could include *CEACAM6*-expressing neutrophils present in colonic samples. (D and E) FACS profiles of the CEABAC normal (non AOM-treated) colon cells (**D**) and colon tumor cells (**E**). Solid line, *CEA* cell surface level; Dotted line, *CEACAM6* cell surface level; Shaded area, background fluorescence from cell membrane. Note that the profiles were smoothed for clear presentation. For normal colon cells (D), mean fluorescence, 8.9 (background), 20.3 (*CEA*) and 15.1 (*CEACAM6*). For colon tumor cells (**E**), mean fluorescence, 10.0 (background), 30.3 (*CEA*) and 135.9 (*CEACAM6*). Subtracting background, this represents 2.7- and 20-fold increases in *CEA* and *CEACAM6* expression, respectively. The average increases in *CEA* and *CEACAM6* expression for five different tumors were 2.1- and 20-fold, respectively.

less in AOM-induced rodent colon tumors (21,22). Here, only one mutation was found at codon 241 (exons 7–8), corresponding to human *p53* codon 244, in a CEABAC tumor (Table II and Figure 4).

Due to a small sample size and low mutation frequency, changes of mutation frequency in the *Kras* and *p53* genes in CEABAC mice remained inconclusive. Nevertheless, the 2-fold increase in β-*catenin* mutations seen per mouse may indicate that CEABAC mice are more susceptible to the acquisition and/or retention of gene mutations involved in colorectal cancers.

Discussion

Human GPI-anchored CEACAM family genes, *CEA* and *CEACAM6*, were demonstrated to be instrumental for tumor progression in various *in vitro* model systems (5–10) and animal assays using human and murine cells transfected *in vitro* (7,31,32). However, these tumorigenic effects had not yet been demonstrated *in vivo*, mainly because of the absence of adequate animal models. Mice lack GPI-anchored CEACAM family members and, by supplementing the mouse genome with a part of the human CEACAM family gene locus

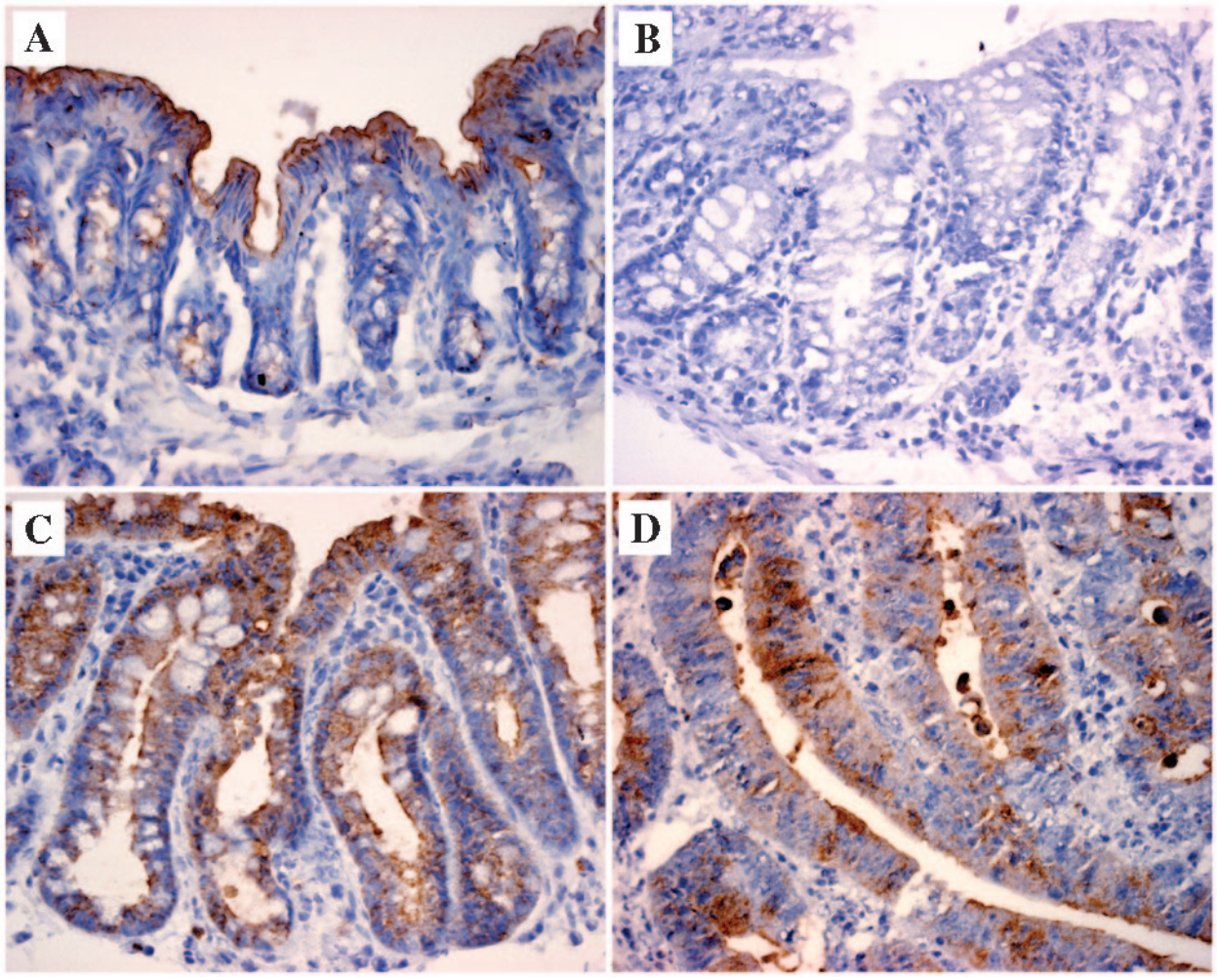

Fig. 3. Immunohistochemistry for human CEACAM family gene expression. Cryosections were stained with polyclonal rabbit anti-CEA antibody, which detects all human CEACAM family members, and counterstained with hematoxylin (×400). (**A**) Normal colonic epithelium from an untreated CEABAC10 mouse. Human CEACAM family members are localized mainly on the apical surfaces of colonocytes towards the top of the crypts and on the mucosal surface. (**B**) Normal epithelium adjacent to a colon tumor from an AOM-treated WT mouse. No staining was present as expected. (**C**) 'Normal' epithelium adjacent to colon tumors from AOM-treated CEABAC10 mice. Human CEACAM family members are expressed throughout the crypts, in contrast to untreated epithelium (A), thus showing a field effect due to the adjacent tumors. (**D**) AOM-induced colon tumor obtained from a CEABAC10 mouse.

containing *CEA*, *CEACAM6* and *CEACAM7* genes by means of BAC transgenesis, human-like colonic expression of *CEA*, *CEACAM6* and *CEACAM7* was obtained in the CEABAC transgenic mouse colon (16); transmembrane *CEACAM1* was also expressed in mouse colon from the endogenous *murine* gene. As in the WT FVB mice, neither tumors nor aberrant crypt foci, precursor lesions for colon tumors (33), could be found spontaneously in colons from the CEABAC mice at any age (16), as was previously reported for *CEA*-only transgenic mice (13,14). This may not be surprising since, in our case at least, the expression pattern of these human CEACAM family genes within the colonic tissue was normal, i.e. minimal surface expression in undifferentiated epithelial cells with proliferative capacity at the bottom of colonic crypts, gradually increasing expression in differentiating cells in the middle, and highest expression in fully differentiated cells on the top of the crypts (16,23). Overexpression of *CEA* and *CEACAM6* in cells with division potential prior to their differentiation has been hypothesized to lead to tumorigenic phenotypes *in vivo* (7,9). A more spatially uniform and intracellular cryptal expression of CEA as seen by immunohistochemistry was reported for *CEA*-only transgenics made by Eades-Perner *et al.* (14). However, this pattern was not observed in the transgenic mouse made with the same transgene by Clarke *et al.* (13). Thus, we suggest that this abnormal staining pattern was possibly due to the extremely high sensitivity of *CEA* detection achieved by these workers, resulting in higher apparent levels in the lower proliferative regions of the crypts and saturated levels in the upper differentiated regions.

In this study, the CEABAC mice were shown to have a highly significantly increased susceptibility to AOM-induced colon tumor formation relative to WT FVB mice, which are known to produce colorectal adenocarcinomas after AOM treatment (25). Since increased susceptibility was observed in both transgenic lines, the presence of the human CEACAM family genes, rather than a rare insertional mutation due to transgene integration, was apparently responsible for the phenotype. These results, in concordance with previous *in vitro* studies reported by different groups (5–10), strongly support a tumorigenic role for human GPI-anchored CEACAM family members *in vivo*. Thompson *et al.* (15)

Table II. *β-Catenin, Kras* and *p53* mutations in AOM-induced tumors

Gene	Exon number	Codon number[a]	Number of mutations (%)[b]		Nucleic acid change	Amino acid change
			WT	CEABAC		
β-Catenin	3	32	1 (10)	3 (14)	GAT→AAT	Asp→Asn
		33	1 (10)	2 (10)	TCT→TTT	Ser→Phe
		34	5 (50)	14 (67)	GGA→GAA/AGA[c]	Gly→Glu/Arg[c]
		41	3 (30)	2 (10)	ACC→ATC	Thr→Ile
		Total[d]	10 (53)	21 (64)		
		Avg no./mouse[e]	3.3	7.0		
Kras	1	12	1 (100)	1 (50)	GGT→AGT/GAT[f]	Gly→Ser/Asp[f]
		13	0 (0)	1 (50)	GGC→GAC	Gly→Asp
		Total[d]	1 (5)	2 (6)		
		Avg no./mouse[e]	0.33	0.67		
p53	5–6		0 (0)[g]	0 (0)[g]		
	7–8	241	0 (0)	1 (100)	GGG→AGG	Gly→Arg
		Total[d]	0 (0)	1 (3)		
		Avg no./mouse[e]	0	0.33		

[a]Codons where mutations were found.
[b]Mutations in tumors >1 mm^3 collected from 3 WT and 3 CEABAC mice (19 WT and 33 CEABAC tumors). Number shown in parenthesis for 'total' denotes the percentage of tumors with mutations, whereas those for individual codons denote the percentages of total number of mutations.
[c]GGA → AGA (Gly → Arg) mutation was found in one CEABAC tumor.
[d]Total number of mutations found in three WT and three CEABAC mice.
[e]verage number of mutations found in each mouse.
[f]GGT → GAT (Gly → Asp) mutation was found in CEABAC tumor and GGT → AGT (Gly → Ser) mutation in WT tumor.
[g]Due to excessive sequencing background in exons 5–6 of 3 WT and 8 CEABAC tumor DNA samples, only results from 16 WT and 25 CEABAC tumors are shown.

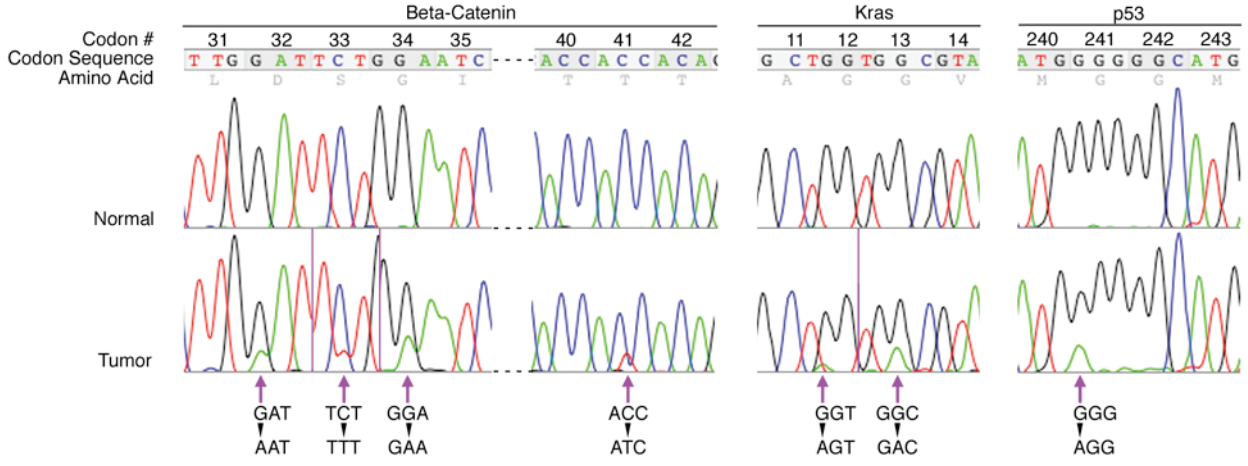

Fig. 4. Mutations of *β-catenin*, *Kras* and *p53* induced by AOM. Codon numbers, normal codon sequences and corresponding amino acid sequences are shown on the top panel. Chromatograms of normal and mutated sequences are shown in the middle and bottom panels, respectively. DNA sequences: A, green line; G, black line; C, blue line; T, red line). Point mutations are shown by purple arrows with corresponding mutations. Four point mutations are shown for *β-catenin* (codon 32, GAT→AAT, D32N; codon 33, TCT→TTT, S33F; codon 34, GGA→GAA, G34E; codon 41, ACC→ATC, T41I), 2 for *Kras* (codon 12, GGT→AGT, G12S; codon 13, GGC→GAC, G13D) and codon 1 for *p53* (codon 241, GGG→AGG, G241R). Note that the point mutations shown were present in different tumors and that the higher the mutant peak the higher the proportion of tumor cells bearing that mutation, suggesting earlier acquisition of the mutation (the mutant peak reaches a theoretical maximum, i.e. 100% of tumor cells have one allele mutated, when WT and mutant peaks are equal in height). Amino acid abbreviations: A, Alanine; D, asparatic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; L, lysine; M, methionine; N, asparagines; R, arginine; S, serine; T, threonine; V, valine.

however, failed to demonstrate any significant tumorigenic effects of *CEA* expression in their *CEA*-only transgenic mice, even when crossed with APC-deficient mice. However, it is difficult to compare our results with those of Thompson *et al.* (15) because tumors formed in the APC$^{Min/+}$ mice are predominantly of intestinal instead of colonic origin. Since the baseline expression of *CEA* is much lower in the small intestine than colon (14,16), it is perhaps not surprising that Thompson *et al.* (15) detected no apparent effect of *CEA* in these mice. Thus, it is unclear at present whether *CEA* expression can synergise with the effect of APC mutations. It would be interesting to treat the *CEA*-only transgenic mice with AOM or to introduce APC mutations into the CEABAC mice.

It is not clear from this work, however, which of the CEACAMs present in the CEABAC are responsible for these effects. Since, as in humans, *CEACAM7* is expressed only at very low levels in these transgenics' colons (16) and underexpressed rather than overexpressed in human colorectal cancers (1), and *CEACAM3* is not at all expressed in the colon

(1,16), it seems likely that *CEA* and/or *CEACAM6* are responsible and, in fact, both of the latter genes have been shown to elicit tumorigenic effects in various model systems (5–10).

Overexpression of *CEA* and *CEACAM6* (2- and 20-fold, respectively) could be shown on the cell surface of colonocytes from the AOM-induced tumors in the CEABAC mice relative to normal colonocytes from untreated CEABAC mice. Similar observations were previously documented for human colon tumors (23). The relative increase in the expression was shown to be much greater for *CEACAM6* than for *CEA*, as has been documented for human colon tumors (23). The question therefore arises as to whether *CEACAM6* could play a stronger role in colon tumorigenesis. Although both *CEA* and *CEACAM6* have been shown to elicit similar tumorigenic effects individually in various model systems (5–10), the possibility of synergism in tumorigenesis has not been previously examined.

Normal-looking epithelium adjacent to the tumors in CEABAC mice showed an abnormal expression pattern of *CEA*/*CEACAM6*, i.e. an absence of the aforementioned expression gradient within the colonic crypts, which has also been observed in human colorectal carcinomas, where it has been attributed to a field effect emanating from the tumor (27). The magnitude of overexpression and abnormal cryptal expression pattern of *CEA*/*CEACAM6* in this study thus mimic very closely these features seen in colonic tumor specimens taken from human patients. To our knowledge, this is the only model system showing the same pattern of changes of CEA family members expression as seen in human colorectal carcinomas.

This de-regulated expression could be due to the following. On the one hand, this could be a primary focal effect at various points in the colon exerted by the AOM treatment on the transcriptional control of *CEA*/*CEACAM6* genes. Promoter hypomethylation of the *CEA* gene leading to transcriptional de-repression has been observed in human colon cancers (34,35). Since AOM induces G:C→A:T conversions, it could theoretically remove CpG methylation sites in the promoter region. On the other hand, the de-regulated expression could be a secondary effect exerted by the developing tumor. It is known that certain cytokines, such as IFN-γ, IFN-α and IL-6, can increase *CEA* and *CEACAM6* expression in various human colon cancer cell lines *in vitro* (36–38) and these cytokines could be secreted in the tumor microenvironment as a consequence of normal immune reaction towards the developing tumors (39). In any case, deregulated overexpression of *CEA*/*CEACAM6* could accelerate tumor formation in the CEABAC mice, given the tumorigenic effects of *CEA*/*CEACAM6* overexpression observed in other experimental systems (5–10).

Adaptive gene mutations unarguably contribute to tumor progression after AOM treatment. But the question arises as to how the colonic expression of GPI-anchored CEACAM family genes enhances the action of AOM, i.e. increases the frequency of tumorigenic mutations, at least those in the β-*catenin* gene, in the CEABAC mice. *CEA* overexpression has been shown by Screaton *et al.* (32) to enhance cellular transformation in rat myoblasts expressing *v-Myc* and *Bcl-2*, two well-known oncogenes. These authors showed that in such cells, *CEA* can induce a markedly increased frequency of heritable lesions conferring anchorage-independent growth (32). The exact nature of these mutations was not determined, but *CEA* was shown to promote cellular transformation and to act as a carcinogen (32). Similarly, in the present case, the expression of GPI-anchored CEACAM family genes could lower the threshold in CEABAC colonocytes for the acquisition and/or retention of transforming mutations after AOM treatment.

Apart from the increased incidence of gene mutations in the CEABAC mice, a shift of mutational spectrum of β-*catenin* was also observed, i.e. from codon 41 to codons 32/34. Opposite shifts were documented previously in rats given a single dose of chlorophyllin after a short-term DMH treatment (40) and in rats with a long-term DMH treatment (41). Although mutations in all those residues were shown to reduce phosphorylation, ubiquitination and degradation of β-*catenin*, they may not be functionally equivalent (41–45). Thus, it would be interesting to further investigate the functional differences between these mutants and the selective pressure for D32N and G34E over T41I mutations in the presence of GPI-anchored CEACAM family genes after AOM treatment in mice.

In concordance with other studies, the mutational spectrum of β-*catenin*, *Kras* and *p53* genes in AOM-induced mouse tumors was found to be quite different from that of human colorectal cancers, in which the mutation frequency is much lower in β-*catenin* and much higher in *Kras* and *p53* genes (22,28–30). This apparent difference could be related to the experimental animal species utilized. Mice may not tolerate the detrimental effects of *Kras* and *p53* mutations as well as humans so that colonocytes acquiring these mutations could be eliminated before they can form visible lesions (46). Alternatively, the functionality of these gene mutations could be affected by 'modifier' genes which are species-specific. The tumorigenicity of β-*catenin* mutations could be augmented in mice and therefore positively selected. Regardless of the actual mechanism, future work should attempt to make these AOM models more human-like.

In conclusion, our results strongly support a positive role for the human GPI-anchored CEACAM family genes, *CEA* and *CEACAM6* (and possibly *CEACAM7*), in tumor formation *in vivo*. Thus, these genes should not only be considered as tumor markers, but also as cancer susceptibility genes. Understanding the cellular and molecular basis for this increased susceptibility could be important to the field of cancer biology and therapeutics, given the high proportion of human cancers showing overexpression of these molecules. In addition, the similarity of the de-regulation of *CEA*/*CEACAM6* expression seen in AOM-treated CEABAC mice and human cancers makes the CEABAC mice a unique animal model system for the development and pre-clinical testing of the CEA-based therapies.

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