

# Identification of gene expression profiles correlated to tumor progression in a preclinical model of colon carcinogenesis

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**Abstract.** The rat azoxymethane (AOM)-induced colon carcinogenesis model provides useful information for understanding human colorectal neoplasia. Here, we used the AOM model to measure the gene expression profiles of biomarkers related to tumor progression. We assessed tumor progression stages by computed tomographic (CT) colonography. Messenger RNAs were isolated from tumors and mucosal samples, and gene expression levels were assessed by real-time quantitative polymerase chain reaction (PCR). We show that early stages of tumor progression are associated with an upregulation of matrix metalloproteinase-7 (MMP-7) and of genes involved in the inflammatory response, including interleukin (IL1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ). The ratio of B-cell lymphoma/leukemia 2 (Bcl-2)-associated X proteins (Bax) to Bcl-2 transcript (proapoptotic/antiapoptotic signals) is elevated in early stages of tumor progression (Bax/Bcl-2 >1) and reversed in more advanced stages of tumor development (Bax/Bcl-2 <1). These changes are associated with the reduced expression of TNF-related apoptosis-inducing ligand (TRAIL)-death receptor 5 (DR5) and FAS (also known as CD95) apoptotic receptors. Advanced stages of tumor development are characterized by an increase in MMP-9 expression associated with the upregulation of components of the innate immune system:  $\alpha$ -defensin 5 (DEF-5) and neutrophil gelatinase-associated lipocalin (NGAL). The identification of specific gene expression profiles that correlate with tumor progression stages, as reported in the present study, may represent an important step in evaluating potential chemopreventive and/or chemotherapeutic agents prior to initiating clinical trials.

## Introduction

A great majority of colorectal cancers are thought to arise from a pre-existing polyp through the accumulation of successive genetic alterations. A growth phase over many years is necessary for the transformation of a small initial polyp (adenoma) into a malignant tumor (carcinoma) (1-4). Unfortunately, the factors that determine which particular adenoma will increase in size, how quickly it will grow, and whether an individual lesion is at greater risk of developing into a carcinoma than another are not known. As a result, there is a need for reliable preclinical models to examine cellular and molecular mechanisms related to the human disease to better understand the development of colon tumors. Animal models have contributed significantly to our understanding of the carcinogenic process and of the multiple environmental factors involved in the pathogenesis of colon cancer (5,6). These animal models include the induction of colorectal tumors in rats by the administration of the chemical carcinogen azoxymethane (AOM). AOM-induced carcinogenesis has been used by many investigators to initiate tumor formation and to study the effects of chemopreventive agents on colon carcinogenesis (5,6). AOM initiates a multistep carcinogenic process that transforms normal colonic epithelia into a carcinoma with an adenomatous polyp as an intermediate step in the process (7). Tumors induced by AOM share many histopathologic and genetic characteristics with sporadic human colon tumors (6).

In humans, colon carcinogenesis is a long, chronic process that is thought to take place over 10 to 20 years (8). In the AOM-induced rat model, polyps may be detected as early as five months and adenocarcinoma by eight months, making the AOM model very attractive for understanding colorectal neoplasia in man (9). Using the AOM model we have recently established a reliable procedure allowing us to follow tumor development by computed tomographic (CT) colonography in living rats. CT colonography allows for the assessment of the efficacy of chemopreventive agents (10). The development and progression of colon carcinogenesis in both humans and rodents are known to be caused by the accumulation of cancer-related genetic alterations, resulting in altered gene expression of oncogenes, tumor suppressor genes, and mismatch repair genes (11,12). These alterations affect the expression of a variety of downstream genes, including those involved in

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regulating the cell cycle, apoptosis, adhesion, and angiogenesis (13).

To gain more insight into the molecular mechanisms involved in the progression of small polyps into malignant tumors, we measured the gene expression profile of several potential biomarkers related to tumor progression after colon cancer initiation by AOM. We used three-dimensional (3D)-CT colonography to identify rats exhibiting tumors at different stages of progression, and to measure the tumor volumes *in situ*. This approach allowed us to examine changes in the mRNA profiles of several genes from small polyps to adenocarcinoma. We examined the phenotypic expression of inflammatory genes (including interleukin- $\beta$  (IL1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and the matrix metalloproteinases-7 and -9 (MMP-7 and MMP-9), immune defense genes including neutrophil gelatinase-associated lipocalin (NGAL or lipocalin 2) and  $\alpha$ -defensin-5 (DEF-5), and several genes involved in apoptotic or survival responses (death receptor 5 (DR5), TNF-related apoptosis-inducing ligand (TRAIL), FAS receptor (also known as CD95), FAS ligand (FASL), B-cell lymphoma/leukemia 2 (Bcl-2) and Bcl-2 associated X protein (Bax).

## Materials and methods

**Animal model and treatment.** All experiments were performed in accordance with the institutional guidelines of the French Ethics Committee (authorization no. A67-480, French Ministry of Agriculture). Male Wistar rats ( $n=18$ ) were obtained from Charles River Laboratories (Les Oncins, France) and weighed between 240 and 250 g. The rats were housed under standardized conditions (22°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes/h) and fed a standard diet with free access to drinking water. One group of 12 animals received intraperitoneal injections of AOM (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 15 mg/kg of body weight, once a week for two weeks. A control group of six rats not treated with AOM received only the excipient (0.9% NaCl).

**CT colonography and assessment of tumors.** Animals were prepared for the CT colonography as described previously (10) and colonography was performed using a micro-CT scanner Micro Cat II (Imtek Inc., San Diego, CA, USA). Rats were scanned in a supine position under anesthesia with isoflurane (2-3%) in O<sub>2</sub>/N<sub>2</sub>O (30/70%). Bio-monitoring during acquisition allowed for the synchronization with an automated breath tracking system to avoid breathing movement interferences. The acquisition duration was 10 min for each animal. Parameters were as follows: 500  $\mu$ A, 80 kV, 150  $\mu$ m slice thickness. The micro-scanner differs from a conventional scanner used for humans by the fact that during a scan, two dimensional (2D) X-ray images are acquired as the source (X-ray tube) and the charge-coupled device (CDD) detector completes only one rotation of 360° around the animal. The Microcat™ software allows a 3D reconstruction process from the 2D-X ray images. Axial, sagittal and coronal views are obtained allowing for the measurement of the tumor size *in situ* in individual rats (Fig. 1).

All animals were sacrificed between 8 and 10 months after AOM injection, after the identification and measurement of

tumor volume by CT-colonography, and after determination of the tumor's developmental stage. The entire colon was collected, washed with saline buffer, and cut flat with the mucosa facing up to identify tumors. All tumors were collected and frozen in liquid nitrogen for analyses. Tumor-free mucosal samples of the proximal colon of AOM-treated rats were scraped off with a glass slide and frozen in liquid nitrogen.

Mucosal samples from the NaCl-treated control rats were collected from the proximal colon using the same procedure as described for the AOM-treated rats. The colon of these rats was tumor-free and exhibited no pre-neoplastic lesions (i.e., aberrant crypts).

**Real-time quantitative reverse transcriptase-polymerase chain reaction analyses.** RNA was isolated from the tumors and mucosal scrapings. Total RNA was isolated using an RNeasy Plus Mini kit (Qiagen, Austin, TX, USA). A high capacity cDNA reverse transcription kit (ABI, Foster City, CA, USA) was used for cDNA synthesis as recommended by the supplier. Polymerase chain reaction (PCR) was performed by using ABI TaqMan gene expression assays for MMP-7, MMP-9 (assay ID: Rn00563467; Rn00560661), IL1 $\beta$ , TNF $\alpha$  (assay ID: Rn99999009; Rn99999017), and Bcl-2, Bax, DEF-5, FAS(CD95), FASL, DR5, TRAIL, NGAL (assay ID: RN99999125; RN02532082; RN01478512; RN00685720; RN00563754; RN00686175; RN01753393; RN00590612) according to the manufacturer's instructions. All samples were run in triplicate in a 25  $\mu$ l reaction volume. Real-time quantitative PCR was performed by using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems Sequence detector) in triplicate wells. The data were analyzed by a comparative threshold cycle ( $C_T$ ) method.  $C_T$  values were calculated using the 7500 SDS software (Applied Biosystems). The corresponding mRNA level from colonic mucosa of NaCl-treated control rats was used in all determinations as an external reference, and the level of  $\beta$ -actin mRNA (assay ID: Rn00667869) of each sample was used as an internal reference to normalize the data. The mRNA fold changes (mRNA relative expression) were expressed relative to the corresponding mRNA mean value found in the mucosa of the NaCl-treated control rats (obtained 8 and 10 months after NaCl injection) and were calculated using the 2<sup>-DDC<sub>T</sub></sup> method (14).

**Statistical analysis.** Data are reported as the mean value  $\pm$  SE. Statistical differences were evaluated by one-way analysis of variance (ANOVA) and specific differences between groups were identified using the Student Neuman-Keuls multiple comparison test.

## Results

A total of 33 tumors were detected by CT colonography in the AOM-treated rats ( $n=12$ ) and all were confirmed at autopsy. In the colon of several rats, we observed that multiple tumors with different maturation stages were simultaneously present. For example, as shown in Fig. 1, the colon of one animal exhibited three tumors measuring 5, 40 and 300 mm<sup>3</sup>. All of the collected tumors were distributed in function based on

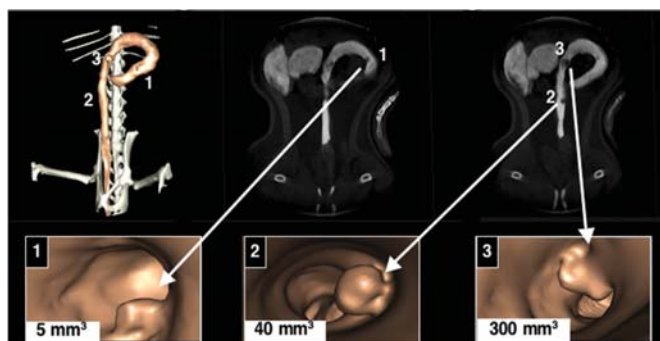


Figure 1. Representative anterior CT colonographic coronal views and the corresponding 3D-reconstruction obtained from an anesthetized rat eight months after AOM injection. This rat possessed three tumors all at different developmental stages. Numbers and arrows indicate the respective localization of the tumors detected by CT colonography.

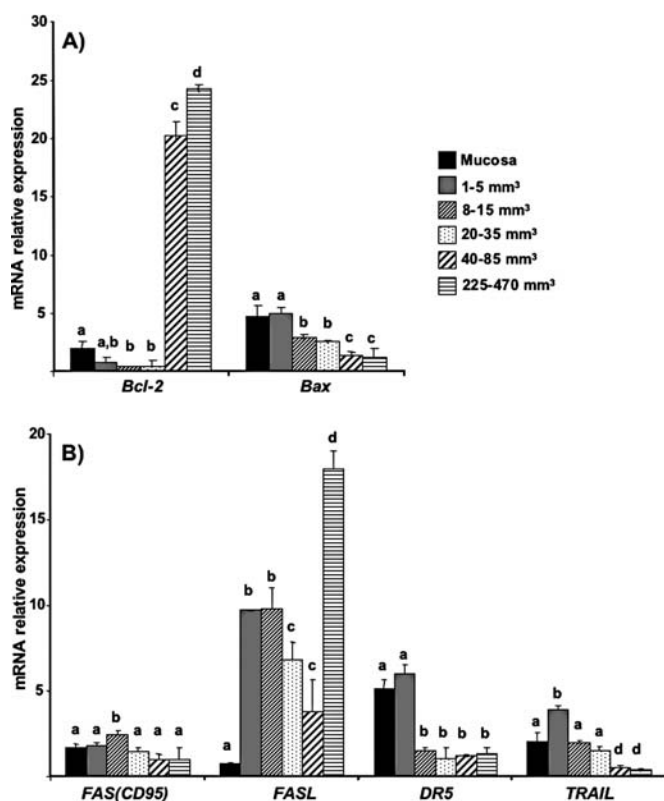


Figure 3. Changes in the mRNA expression levels of (A) Bcl-2 and Bax, and (B) components of the extrinsic apoptotic pathway: FAS(CD95), FASL, DR5, and TRAIL. For further details refer to the legend of Fig. 2. The data are presented as the mean  $\pm$  SE (n=12 for AOM-rats, and n=6 for NaCl-treated rats). For each gene transcript, columns not sharing the same superscript letter differ significantly,  $P < 0.05$ .

their size in five different groups: small polyps ranging from 1 to 5 mm<sup>3</sup> (n=7), tumors between 8 and 15 mm<sup>3</sup> (n=4), tumors ranging from 20 to 35 mm<sup>3</sup> (n=5), tumors ranging from 40 to 85 mm<sup>3</sup> (n=9), and the most developed tumors ranging from 225 to 470 mm<sup>3</sup> (n=8). For each group, we determined the expression level of inflammatory genes (including IL1 $\beta$ , TNF $\alpha$ , MMP-7, MMP-9), innate immunity genes (including DEF-5 and NGAL), and genes involved in cell survival and

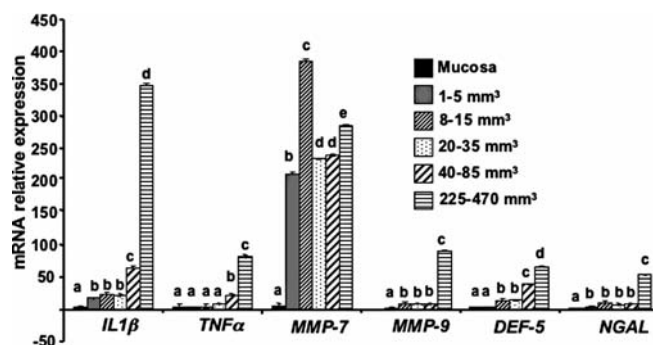


Figure 2. Changes in the mRNA expression level of pro-inflammatory cytokines (IL1 $\beta$  and TNF $\alpha$ ), matrix metalloproteinases (MMP-7 and MMP-9), and components of the innate immune response (DEF-5 and NGAL). The mRNA was obtained from mucosal and tumor samples at various stages of tumor development from the colon of AOM-treated rats. The collected tumors were distributed based on their size into five different groups: small polyps ranging from 1 to 5 mm<sup>3</sup> (n=7), tumors with sizes ranging from 8 to 15 mm<sup>3</sup> (n=4), 20 to 35 mm<sup>3</sup> (n=5), 40 to 85 mm<sup>3</sup> (n=9), and larger tumors ranging in size from 225 to 470 mm<sup>3</sup> (n=8). Real-time quantitative PCR was performed in triplicate wells. The corresponding mRNA level from colonic mucosa of NaCl-treated control rats was used in all determinations as an external reference, and the level of  $\beta$ -actin mRNA of each sample was used as an internal reference to normalize the data. The mRNA fold changes (mRNA relative expression) were expressed relative to the corresponding mRNA mean value found in the mucosa of NaCl-treated control rats (obtained 8 and 10 months after NaCl injection) and were calculated using the  $2^{-\Delta\Delta C_t}$  method (14). Data are presented as the mean  $\pm$  SE (n=12 for AOM-rats and, n=6 for NaCl-treated rats). For each gene transcript, columns not sharing the same superscript letter differ significantly,  $P < 0.05$ .

cell death (including Bcl-2, Bax, FAS(CD95), FASL, DR5, TRAIL) by real-time quantitative PCR.

When compared to normal colonic mucosa of NaCl-treated control rats, AOM-treatment initiated the up-regulation of IL1 $\beta$  and TNF $\alpha$  in the mucosal samples by 3.5- and 5-fold, respectively (Fig. 2). Compared to the level found in the mucosal samples of the AOM-treated rats, a 20-fold increase in expression of IL1 $\beta$  was observed in the group of smaller sized tumors (ranging from 1 to 35 mm<sup>3</sup>), and the expression of IL1 $\beta$  increased as the tumor size increased: +65-fold in tumors ranging from 40 to 85 mm<sup>3</sup> and +350-fold in the more advanced tumors (225-470 mm<sup>3</sup>). A similar trend was observed for TNF $\alpha$  expression with the highest expression (+80-fold) in the largest most advanced tumors (225-470 mm<sup>3</sup>). In humans, an increased expression of matrix metalloproteinases (MMP) genes has been associated with early stages of tumor development (15). Here, we observe that MMP-7 gene expression was already enhanced in small polyps (+210-fold) compared to the level found in the mucosal samples. The level of MMP-7 gene expression remained high during tumor progression (Fig. 2). In contrast, the expression pattern of MMP-9 was similar to the expression pattern of IL1 $\beta$  and TNF $\alpha$ . The highest expression level of MMP-9 was present in the more advanced tumors (+90-fold).

We examined the expression profiles of two components of innate immunity, NGAL and DEF-5. DEF-5 was not upregulated in small polyps (1 to 5 mm<sup>3</sup>) compared to the mucosal samples of AOM-treated rats but exhibited a progressive increase in expression as the tumor size increased (from +13- to



+40-fold in 8 to 85 mm<sup>3</sup> tumors, respectively), reaching a maximum of +66-fold in the more advanced tumors. However, the expression of NGAL remained low in the early stages of tumor development and was only upregulated (+55-fold) in the more advanced tumors (Fig. 2).

Tumor progression correlates with the expression of various factors controlling cell survival and/or cell death. The colonic mucosa of AOM-treated rats exhibited an upregulation of both pro-apoptotic [FAS(CD95), DR5, FASL, TRAIL and Bax] and anti-apoptotic (Bcl-2) genes. The major effect was on Bax and TRAIL-DR5 genes. Both Bax and TRAIL-DR5 were enhanced 5-fold compared to normal mucosa (Fig. 3). At early stages of tumor progression in groups with tumor sizes of 1-5 mm<sup>3</sup>, 8-15 mm<sup>3</sup>, and 20-35 mm<sup>3</sup>, the Bax/ Bcl-2 transcript ratio remained high (>1), suggesting the possibility that these tumor cells are undergoing apoptosis. In contrast, at later stages of tumor progression, in groups with tumor sizes of 40-85 and 225-470 mm<sup>3</sup>, the Bax/Bcl-2 ratio was reversed (<1). This switch in the Bax/Bcl-2 ratio seemed related to the upregulation of the anti-apoptotic Bcl-2 gene (+20/25-fold) and to the parallel downregulation of Bax expression. These data suggest that the survival pathway is favored in these tumor cells. This conclusion is supported by the downregulation of FAS(CD95), TRAIL-DR5, and TRAIL in these tumors. However, FASL gene expression shows a different expression pattern during tumor progression (Fig. 3).

## Discussion

Large-scale screening of colon tumors using cDNA arrays or reverse transcriptase-PCR have identified many genes with altered expression (16-18). However, more often, these changes resulting from progressive genomic alterations in association with the disruption of large metabolic networks have been studied at advanced stages of tumorigenesis. The ability to follow tumor development by CT-colonography after AOM-initiation allowed us to measure tumor sizes in living rats and to collect tumors at various stages of progression. Here, we were able to collect tumors ranging from small polyps (1-5 mm<sup>3</sup>) to large advanced tumors (225-470 mm<sup>3</sup>). To gain more insight into the molecular mechanisms involved in the progression of small polyps to more advanced tumors, we followed the expression of specific genes known to be involved in inflammatory and innate immune responses, as well as genes involved in the control of cell death and/or survival.

Herein, we report that pro-inflammatory genes (TNF $\alpha$ , IL1 $\beta$ , MMP-7, and MMP-9) are upregulated during tumor progression. The observation that TNF $\alpha$  and IL1 $\beta$  expression progressively increased during tumorigenesis is in agreement with previous data reporting that the genes for inflammatory cytokines are highly expressed in malignant colorectal tumors (19). The key role of these genes in tumor formation was demonstrated in a study showing that the transgenic over-expression of IL1 $\beta$  in gastric mucosa was sufficient to induce gastric cancer in mice (20).

MMPs play an important role in the recruitment of inflammatory cells to the tumor by contributing to the disruption of extracellular matrix and cell junctions. Although the tumor stroma cells produce most of the MMPs whose levels are increased in the colon cancer (i.e., MMP-2 and MMP-9), tumor

epithelial cells express another MMP, MMP-7 (21). MMP-7 was reported to be frequently overexpressed in human cancer tissues and to be associated with cancer progression, invasion and metastasis (22). Our data indicate that MMP-7 is upregulated at early stages of tumor development and this high level of expression is maintained throughout tumorigenesis. In contrast, MMP-9 is only upregulated at later stages of tumor progression. These data are in accordance with a critical role for MMP-9 in the metastatic progression of colon cancer (23). Our data also support the hypothesis of separate roles for MMP-7 and MMP-9 in the development of colon carcinomas with MMP-7 being involved in the initial steps of tumor development and MMP-9 being expressed by more advanced tumors, favoring invasion and metastatic spread (24). TNF $\alpha$  is known to contribute to malignant cell invasion by stimulating MMP-9 gene expression (25). Thus, MMPs may mediate a positive feedback loop in the exacerbated inflammatory network that promotes tumor progression, which may explain the higher observed expression of inflammatory cytokine genes in the later stages of tumor development.

Two components of the innate immune system DEF-5 and NGAL, exhibited different patterns of expression during tumor growth. DEF-5 was upregulated in small polyps and exhibited a further progressive increase during tumorigenesis. In contrast, NGAL was only significantly upregulated at later stages of tumor development.

The presence of abnormally high expression levels of  $\alpha$ -defensins has been identified by proteomic analysis in a variety of human tumors (26). These defensins were shown to be expressed in the cytoplasm of the tumor cell and at the cell surface. Mitogenic effects of defensins have been previously described in mouse fibroblasts and epithelial cells (27,28). However, the molecular mechanisms involved in the overexpression of these peptides and their role in carcinogenesis remain unknown. Alternatively, NGAL has been associated with metastatic stages of tumorigenesis. The role of NGAL in tumor formation may be linked to the fact that NGAL stabilizes the enzymatic activity of MMP-9 (29,30). For example, NGAL was suggested to play an important role in breast cancer by protecting MMP-9 from degradation and thereby favoring tumor invasion (31). The data presented here shows a good correlation between the level of MMP-9 and NGAL genetic expression in the more advanced tumors.

Tumor progression correlates with the expression of genes involved in regulating cell survival and/or cell death. Therefore we measured the expression level of genes involved in the control of the two main cell death pathways: the extrinsic apoptotic pathway triggered by specific death receptors located at the cell surface such as FAS(CD95) or TRAIL-DR5, and the intrinsic apoptotic pathway induced after mitochondrial injury. The intrinsic pathway is controlled by members of the Bcl-2 family, including the anti-apoptotic Bcl-2 and the pro-apoptotic Bax protein. The data presented here show that Bcl-2 gene expression is downregulated at early stages of tumor development as compared to the level of Bcl-2 found in the mucosal samples of AOM-treated rats. Simultaneously, a high level of Bax expression was maintained in these tumors. These data indicate a possible preservation of apoptotic signaling at this stage. These data are also supported by the concomitant early upregulation of FAS(CD95) and TRAIL-death receptor

and ligand genes during tumorigenesis. Of note, the higher Bax/Bcl-2 transcript ratio was reversed by a huge increase in Bcl-2 and a drop in Bax expression (Bax/Bcl-2 ratio  $<1$ ) in later stages of tumor development. These data suggest that the Bax/Bcl-2 transcript ratio may represent a good marker for the progression of colon tumorigenesis (32). The drop in Bax/Bcl-2 transcript ratio corresponding to the anti-apoptotic signal correlated with a parallel drop in TRAIL-DR5 and FAS (CD95) death receptor transcripts, suggesting that these tumor cells may have a reduced ability to undergo apoptosis.

The data presented here indicate that a high expression of FASL transcripts in more advanced stage of tumor development. These data are in accordance with previously published observations showing that a variety of tumor cells also express FASL, raising the possibility that FASL may enable the tumor cells to counter-attack the anti-tumor immune effector cells with one of their own apoptosis triggers (33). Thus, the observed high level of FASL expression in the more developed tumors may support a role for this ligand in the immune evasion of malignant cells, as well as their metastatic spread (34). Alternatively, MMP-7 has been reported to be able to modulate FASL expression and activation. MMP-7 may generate the release of soluble forms of FASL by cleaving the membrane form of FASL (35). Consequently, the induction of apoptosis by FAS (CD95) activation might be blocked by MMP-7 activity. In this regard, we report that there is a good correlation between the level of FASL and MMP-7 gene expression in more advanced tumors. However, the role of FASL in the immune evasion of tumor cells remains controversial. Recent data suggest that FAS signaling in colon cancer is multifaceted and includes both anti-apoptotic and proliferative signaling in the tumor cells and may favor apoptosis in the normal tissues into which cancer cells metastasize (34).

In the present study, we show that, early stages of tumor progression are associated with an important up-regulation of the MMP-7 gene that is associated with a progressive increase in the expression of genes involved in inflammatory responses, such as IL1 $\beta$  and TNF $\alpha$ . These early stages of tumorigenesis are characterized by an increase in the expression of genes involved in apoptotic signaling, such as TRAIL, TRAIL-DR5, and FAS (CD95). In addition, changes in the Bax/Bcl-2 transcript ratio (proapoptotic signal/antiapoptotic signal) may also represent a good indicator of tumor progression because this ratio is elevated (Bax/Bcl-2  $>1$ ) in the early stages of tumorigenesis and is reversed (Bax/Bcl-2  $<1$ ) at more advanced stages of tumor development. These changes in the Bax/Bcl-2 ratio were associated with the reduced expression of the apoptotic receptors TRAIL-DR5, and FAS (CD95). Advanced stages of tumor development are also characterized by an increase in MMP-9 expression associated with the upregulation of components of the innate immune system (DEF-5 and NGAL).

To identify new preventive or therapeutic approaches, it is important to take into account the mechanisms underlying the progression of colon carcinogenesis in the AOM-initiated rat model. Thus, the identification of specific gene expression profiles that correlate with tumor progression stages, as reported in the present study, may represent an important step in evaluating potential chemopreventive and/or chemotherapeutic agents prior to initiating clinical trials.

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