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Introduction

Colorectal cancer is one of the most common cancers in the world. To discover novel therapeutic strategies, the mechanisms of colorectal cancer development should be understood. In contrast with other cancers, colorectal cancer develops in a unique organ that harbors a broad spectrum of autoimmune diseases (1). Accumulating evidence has indicated a crucial role of toll-like receptor (TLR) signaling in the interaction between luminal microbes and intestinal tumorigenesis (2–4).

TAM tyrosine kinase receptors (Tyr3, Axl and Mer) exert pleiotropic effects (5). Recent studies have shown that TAM receptors inhibit TLR-mediated inflammatory responses in dendritic cells and macrophages (6). Tyr3+/Axl+/Mer− triple knockout mice exhibit a broad spectrum of autoimmune diseases (7). TAM receptors share two humoral ligands: protein S and growth arrest-specific gene 6 (Gas6) (8,9). Protein S is a well-defined vitamin-K-dependent plasma glycoprotein that serves as a cofactor of protein C in the inactivation of factors Va and VIIa in the anticoagulation pathway. Gas6 is a γ-carboxyglutamic acid domain-containing protein that was originally described in growth-arrested fibroblasts (10,11). Gas6 has the same domain organization as, and 43% amino acid identity with, protein S, and has the potential to induce platelet-mediated thrombosis. Both protein S and Gas6 effectively inhibit TLR-induced cytokine production in mouse dendritic cells and macrophages (5,6,12,13). For example, TAM activation by Gas6 inhibits production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, induced by TLR 3, 4 and 9 (6). Taken together with the recent findings regarding the role of innate immunity in intestinal tumorigenesis (2–4), it may be beneficial to investigate the potential involvement of TAM receptors and their ligands in intestinal tumor progression.

Among TAM receptors and two ligands, Axl and Gas6 have been particularly focused on in the field of tumor biology. Axl is associated with clinical outcome of leukemia, melanoma and lung, prostate, ovarian, renal, thyroid and gastric cancers (14–18). Axl has transforming effects on human cancer cell lines (19,20). In contrast, although several reports have demonstrated the role of Gas6 in tumorigenesis by using cancer cell lines (15,16,21), the in vivo role of Gas6 in mouse tumor development or human colorectal cancer progression remains to be elucidated.

In this study, we sought to investigate the role of Gas6 during tumorigenesis in vivo intestines using Gas6+/+ and Gas6−/− mice.

Materials and methods

Cell culture and reagents

Human colorectal cancer cell lines (SW480 and HT29) and a human macrophage cell line (THP-1) were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum. Reconstituted human Gas6, a γ-glutamyl carboxylated active form, was purchased from R&D Systems (Minneapolis, MN).

Cell proliferation assay

SW480 and HT29 were seeded at a density of 2.5 × 104 cells per well in 96-well tissue culture plates; after 24 h, the medium was changed to fetal calf serum-free medium. After 24 h of incubation under the indicated conditions, cell proliferations were measured using the MTS assay (Cell Counting Kit 8; Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol.

Animal model of colitis and intestinal tumorigenesis

Generation of Gas6 knockout mouse with a C57BL6 background was described previously (22). Apcmin−/− mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed under specific pathogen-free conditions at the animal facilities of Kyoto University. All animal experiments were performed in accordance with institutional guidelines, and the review board of Kyoto University granted an ethical permission for this study. Isolations of mouse epithelial cells and stromal monocytes were described previously (23). To analyze colitis-associated tumorigenesis, mice were injected with a single intraperitoneal dose of azoxymethane (AOM) (12 mg/kg body wt; Sigma, St Louis, MO) at the age of 8 weeks and received 2.0% dextran sulfate sodium (DSS) for 5 days three times every 3 weeks (Supplementary Figure 1A, available at Carcinogenesis Online). To analyze the severity of colitis, mice were killed after the administration of 2.5% DSS for 7 days (Supplementary Figure 1B, available at Carcinogenesis Online).

Real-time quantitative reverse transcription–PCR

Total RNA was extracted using Trizol (Invitrogen). Single-strand complementary DNA was synthesized using Transcriptor First Strand Complementary DNA Synthesis Kit (Roche Applied Science, Basel, Switzerland). Quantitative reverse transcription–PCR (qRT–PCR) was performed using SYBR Green I Master (Roche Applied Science) and Light Cycler 480 (Roche Applied Science). Values are expressed as arbitrary units relative to glyceraldehyde 3-phosphate dehydrogenase. Primer sets used are summarized in Supplementary Table 1, available at Carcinogenesis Online.

Abbreviations:

- AOM, azoxymethane
- BM, bone marrow
- DSS, dextran sulfate sodium
- ELISA, enzyme-linked immunosorbent assay
- Gas, growth arrest-specific gene
- mRNA, messenger RNA
- NF-κB, nuclear factor-kappaB
- PMA, phorbol-12-myristate-13-acetate
- SD, standard deviation
- TAM, Tyro3, Axl and Mer
- TLR, toll-like receptor
- TNF, tumor necrosis factor
- qRT–PCR, quantitative reverse transcription–PCR

Inhibitory role of Gas6 in intestinal tumorigenesis

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Colorectal cancer is one of the most common cancers in the world. Among TAM receptors and two ligands, Axl and Gas6 have been particularly focused on in the field of tumor biology. Axl is associated with clinical outcome of leukemia, melanoma and lung, prostate, ovarian, renal, thyroid and gastric cancers (14–18). Axl has transforming effects on human cancer cell lines (19,20). In contrast, although several reports have demonstrated the role of Gas6 in tumorigenesis by using cancer cell lines (15,16,21), the in vivo role of Gas6 in mouse tumor development or human colorectal cancer progression remains to be elucidated.

In this study, we sought to investigate the role of Gas6 during tumorigenesis in vivo intestines using Gas6+/+ and Gas6−/− mice.
Generation of bone marrow chimeric mice

Cell suspensions from male Gas6+/+ and Gas6−/− mouse bone marrow (BM) were prepared from femurs and tibias, filtered and counted. Female or male mice received a single intravenous injection of 1 × 10^7 BM cells, after being lethally irradiated with 10 Gy X-rays. The following groups of chimeric mice were generated: Gas6+/+ to Gas6−/−, Gas6−/− to Gas6+/+, Gas6+/+ to Gas6+/+ and Gas6−/− to Gas6−/− mice. Genomic DNA was extracted from blood 4 weeks later, and BM chimerism was determined by PCR for the Gas6 and Y chromosome–linked Sry genes.

Histological and immunohistochemical analyses

Mouse intestinal tissues were routinely processed. Anti-Tyro3, Axl, Mer, β-actin and phosphorylated p65 (Cell Signaling Technology, Danvers, MA), anti-Gr1 (eBioscience, San Diego, CA), anti-F4/80, Socs3, CD45 and TNF-α (Abcam, Cambridge, MA) and anti-Gas6 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. The primary antibodies were incubated at 4°C overnight, and then secondary antibodies were added. All immunohistochemical analyses were performed with Ig isotype controls or blocking peptides for antibodies.

Enzyme-linked immunosorbent assay

Nuclear protein was extracted from mouse intestinal tissues by using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was measured using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). DNA binding activity of p65 protein was quantified using TransAM Nuclear Factor-kappaB (NF-κB) p65 Transcriptional Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Active Motif).

Clinical sample analysis

Surgically resected specimens were obtained from stage III/IV colorectal cancer patients who had been admitted to Kyoto University Hospital. Written informed consent was obtained from all patients with the protocol approved by the ethics committee of Kyoto University. Patient’s data were anonymously analyzed, and neither the patient nor anyone else could identify the patient with certainty. A total of 62 surgically resected specimens were routinely processed and immunostained. Clinicopathological parameters of those patients are summarized in Supplementary Table 2, available at Carcinogenesis Online. Gas6 staining in human cancer specimens was determined to be ‘high’ when ≥50% of the tumor cells were stained with anti-Gas6 antibody and to be ‘low’ when <50% of the tumor cells were stained with anti-Gas6 antibody. The number of infiltrating CD45-positive leukocytes was counted in randomly selected three high-power fields (×400) in each specimen, and the mean number was determined.

Statistical analysis

Results are presented as the mean values ± standard deviations (SDs) unless otherwise stated. Difference between treatment, group and strains were analyzed using the two-tailed Student’s t-test, chi-square test, Fisher’s exact test, analysis of variance and Bonferroni’s test. Survival rates were estimated by the Kaplan–Meier method and compared using the Gehan–Breslow–Wilcoxon test.
**Results**

Gas6 enhanced the proliferation of colorectal cancer cells and suppressed the activation of macrophages in vitro

Previous reports using cancer cell lines have suggested that Gas6 contributes to tumor progression (15,16,21). First, we examined the effects of Gas6 on epithelial cells and macrophages in vitro. The human colorectal cancer epithelial cell lines, SW480 and HT29 cells, were cultured for 24 h in serum-free medium containing vitamin K (0.5 μM) with or without recombinant human Gas6 protein (0.5 or 5.0 nM). Both cell lines expressed TAM receptors (Figure 1A), and MTS assays showed that the addition of Gas6 protein weakly, but significantly, enhanced the proliferation of both cell lines (Figure 1B). In addition, messenger RNA (mRNA) expression of inflammatory cytokines, such as TNF-α and interleukin-8, was not altered after stimulation with Lipopolysaccharide (LPS) in SW480 and HT29 cells (100 ng/ml; data not shown). Administration of LPS to THP-1 cells, a human macrophage cell line strongly expressing TAM receptors (Figure 1A), pretreated with 100 nM phorbol-12-myristate-13-acetate (PMA) induced the mRNA expression of inflammatory cytokines such as interleukin-8 and TNF-α after 2 h of LPS stimulation (Figure 1C). However, the simultaneous administration of 0.5 nM human Gas6 significantly reduced LPS-induced mRNA expression of TNF-α and interleukin-8. In addition, the mRNA expression of Socs1/3 was significantly enhanced in PMA/LPS-stimulated THP-1 cells after incubation with 0.5 nM recombinant Gas6 for 2 h (Figure 1D). Concomitantly, immunocytochemistry showed that the nuclear accumulation of phosphorylated p65 in PMA/LPS-stimulated THP-1 cells was significantly reduced at 2 h after incubation with 0.5 nM recombinant Gas6 (Figure 1E and F). Thus, Gas6 has an in vitro potential.
Gas6 deficiency enhanced mouse colitis-associated tumorigenesis

Next, we investigated whether Gas6 affects mouse colitis-associated tumorigenesis. Under physiological conditions, the status of epithelial cells, stromal infiltration by inflammatory cells and mRNA expression of TAM receptors were not altered in relation to the Gas6 gene dosage (data not shown). However, after AOM/DSS treatment (Supplementary Figure 1A, available at Carcinogenesis Online), the number of polyps in the Gas6−/− mouse colon was significantly greater than that in the Gas6+/+ mouse colon (Figure 2A and B). Consistent with this, mRNA expression of proliferation cell nuclear antigen in the colonic polyps of the Gas6−/− mice was significantly higher than that in the polyps of the Gas6+/+ mouse (Figure 2C). Infiltration of neutrophils and macrophages into colonic polyps of the Gas6−/− mice was more prominent (Figure 2D). In addition, mRNA expression of inflammatory cytokines, such as TNF-α, CXCL1 and CCL2, was significantly upregulated in the colonic polyps of the Gas6−/− mice (Figure 2E). NF-κB was also significantly activated (phosphorylation of p65) in the colonic polyps of the Gas6−/− mice (Figure 2F). The mRNA expression of representative tumor-promoting factors, such as c-Myc and Cox2 (Pgr2), was significantly upregulated in the colonic polyps of the Gas6−/− mice (Figure 2G). After AOM/DSS treatment, survival of the Gas6−/− mice was significantly shorter than that of the Gas6+/+ mice (P < 0.01; Figure 2H). Thus, contrary to in vitro studies, Gas6 played an inhibitor role in colitis-associated tumorigenesis in mice.

Gas6 was increased locally and appeared to suppress immune responses of infiltrating stromal cells in DSS-induced mouse colitis

Persistent colitis is a risk factor for the progression of colonic tumors (24,25). Therefore, it would be possible in the AOM/DSS-treated Gas6−/− mice that inflammatory responses in the stromal cells (e.g. macrophages) played a pivotal role in enhancing polyp formation. To confirm whether Gas6 inhibits mouse colitis in vivo, we administered DSS to the Gas6+/+ and Gas6−/− mice (Supplementary Figure 1B, available at Carcinogenesis Online).

After administration of 2.5% DSS for 7 days, the Gas6−/− mice exhibited more severe colitis than the Gas6+/+ mice. In the Gas6−/− mice, the colonic crypt architecture was almost completely obliterated, with massive stromal infiltration by inflammatory cells, whereas in the Gas6+/+ mice, the crypt architecture was relatively preserved (Figure 3A). The histological damage score (26) and weight losses (right) in DSS-treated Gas6+/+ and Gas6−/− mice (mean ± SD, n = 10 each). Body weight before the administration of DSS was defined as 100%. *P < 0.05. (C) Top: The numbers of Gr-1-positive and F4/80-positive cells in DSS-treated Gas6+/+ (white bar) and Gas6−/− (black bar) mouse colon (mean ± standard deviation × 106, n = 10). Bottom: Immunostainings for Gr-1 and F4/80 in DSS-treated Gas6+/+ and Gas6−/− mouse colons. Bars, 50 μm. (D) RNA was extracted from colonic tissues in DSS-treated Gas6+/+ (white bar) and Gas6−/− (black bar) mice, and mRNA expression of each factor was evaluated by qRT–PCR in colon tissues of DSS-treated Gas6+/+ and Gas6−/− mice (mean ± SD, n = 10 each). *P < 0.05.

Infiltration of neutrophils and macrophages into colon cancer cells and to suppress the activation of immune responses in macrophages.

Activated Staining of Socs3 and phosphorylated p65 was only partially observed in the non-inflamed Gas6+/+ and Gas6−/− mouse colons (Figure 4C, ascending, arrowheads). In contrast, Socs3 and phosphorylated p65 were vigorously observed in mononuclear cells infiltrating the stroma especially in the inflamed part of the Gas6−/− mouse colon (Figure 4C, rectum). Because mRNA expression of TAM receptors in stromal monocytes was slightly higher than those in epithelial cells (data not shown), these results suggest that locally increased levels of Gas6 suppresses innate immune responses in DSS-induced colitis by upregulation of Socs1/3 and downregulation of NF-κB in infiltrating stromal cells.

Gas6 from both epithelial cells and BM-derived cells contributed to suppression of DSS-induced mouse colitis

Previous reports have demonstrated that Gas6 is secreted by various cell types, such as tumor cells, fibroblasts, neutrophils and macrophages (10,21,27). Consistent with this, there was no significant difference in Gas6 mRNA expression between isolated intestinal epithelial cells and stromal monocytes, and immunostaining against Gas6 was ubiquitous in both residual epithelial cells and stromal inflammatory cells in the DSS-treated Gas6+/+ mice (data not shown). These data suggest that both epithelial and infiltrating stromal cells produce Gas6. To determine the cellular sources of Gas6 that
suppressed DSS-induced colitis, we performed BM transplantations using Gas6+/+ and Gas6–/– mice. Under physiological conditions, there was no evident difference in any mice undergoing BM transplantation (data not shown). After administration of 2.5% DSS for 7 days, the Gas6–/– mice transplanted with Gas6+/+ marrow exhibited the most severe colitis, based on histological damage scores and weight loss (Supplementary Figure 3A–C, available at Carcinogenesis Online). The Gas6–/– mice transplanted with Gas6+/+ marrow showed the least severe colitis. Gas6+/+ mice transplanted with Gas6+/+ marrow and Gas6+/+ mice transplanted with Gas6–/– marrow exhibited nearly the same degree of moderately severe colitis. Furthermore, the mRNA expression of inflammatory cytokines, such as TNF-α and CXCL1, was altered in parallel with the histological changes (Supplementary Figure 3D, available at Carcinogenesis Online). Thus, Gas6 from both epithelial cells and BM-derived cells may contribute to suppression of DSS-induced mouse colitis.

**Gas6 deficiency enhanced intestinal tumorigenesis in the ApcMin mice**

Most human colorectal cancers develop in a ‘multistep’ manner without obvious inflammation. Using ApcMin mice, we examined the role of Gas6 in multistep intestinal tumorigenesis. Non-tumorous intestinal mucosa in the ApcMin Gas6+/+ mice does not show histologically detectable inflammation as well as that in the simple severe colitis, based on histological damage scores and weight loss (Supplementary Figure 3A–C, available at Carcinogenesis Online). The ApcMin Gas6+/+ mice transplanted with ApcMin Gas6+/+ marrow showed nearly the same degree of moderately severe colitis. Furthermore, the mRNA expression of inflammatory cytokines, such as TNF-α and CXCL1, was changed in parallel with the histological changes (Supplementary Figure 3D, available at Carcinogenesis Online). Thus, Gas6 from both epithelial cells and BM-derived cells may contribute to suppression of DSS-induced mouse colitis.

**Elevated Gas6 was associated with better prognosis in advanced colorectal cancer patients**

To determine the role of Gas6 in human colorectal cancer, we immunostained surgically resected specimens of 62 colorectal cancer patients (Figure 6A) and compared patients survival. Higher expression of Gas6 in cancer tissues was associated with better survival in stages III (Figure 6B, left) and IV (Figure 6B, right, P < 0.05) and overall advanced colorectal cancer patients (Figure 6C, P < 0.05). Similar to murine models, increased expression of Gas6 was also significantly associated with decreased infiltration by CD45-positive leukocytes and lower expression of TNF-α (Supplementary Table 3, available at Carcinogenesis Online). These results suggest that Gas6 plays an inhibitory role in human colorectal cancer progression and suppression of local inflammatory responses.

**Discussion**

Previous studies have suggested that Gas6 promotes cancer cell progression in vitro (15,16). A previous study demonstrated that Gas6...
produced from BM-derived cells enhances proliferation of subcutaneously transplanted murine colon cancer cells lacking Gas6 (21).

In this study, we also observed that Gas6 weakly, but significantly, enhances proliferation of human cancer cell lines. Importantly, these effects of Gas6 are exerted at cellular levels. The role of Gas6 should be complicated during in vivo intestinal tumorigenesis because the intestines are inhabited by a vast population of microbes that play important roles in intestinal tumorigenesis (1–4). In line with previous studies (5,6,12,13), we also showed that Gas6 suppressed innate immune responses of a human monocyte cell line.

We analyzed AOM/DSS intestinal tumor formation model and revealed that the number of polyps in the Gas6–/– mice was greater than that in the Gas6+/- mice. Persistent colitis is a well-known risk factor for the progression of colonic tumors both in humans and mice (24,25). In this study, because the DSS-treated Gas6–/– mice exhibited more severe colitis than the DSS-treated Gas6+/- mice, it can be reasoned that the absence of Gas6 and its anti-inflammatory activity was responsible for the increased number of polyps in the AOM/DSS-treated Gas6–/– mice. Thus, in contrast with its in vitro behavior, the property of Gas6 to inhibit inflammatory cytokine production (5,6,12,13) appears to exert an important negative effect on mouse intestinal tumorigenesis.

In monocyte lineages, Gas6/TAM signaling suppresses NF-κB activation and inflammation through the induction of Socs1/3 (5,6). Our BM transplantation experiments showed that Gas6 from both epithelial cells and BM-derived cells contributed to the suppression of

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**Fig. 5.** Deficiency of Gas6 promoted intestinal polyp formation in ApcMin mice. (A) Numbers of the intestinal polyps in ApcMin Gas6+/- and ApcMin Gas6–/– mice (mean ± SD/mouse, n = 10 each). *P < 0.05. (B) Numbers of the intestinal polyps in indicated size fractions of ApcMin Gas6+/- and ApcMin Gas6–/– mice (mean ± SD/mouse, n = 10 each). *P < 0.05. (C) RNA was extracted from small intestinal polyps in ApcMin Gas6+/- and ApcMin Gas6–/– mice, and mRNA expression was evaluated by qRT-PCR (mean ± SD, n = 10 each). *P < 0.05. (D) Nuclear protein was extracted from small intestinal polyps in ApcMin Gas6+/- and ApcMin Gas6–/– mice, and phosphorylation of p65 protein was quantified by ELISA (mean ± SD, n = 5 each). *P < 0.05. (E) Hemoglobin concentrations in ApcMin Gas6+/- and ApcMin Gas6–/– mice were depicted at the indicated time points (mean ± SD/mouse, n = 5 each). *P < 0.05. (F) There was a significant difference of survival between ApcMin Gas6+/- and ApcMin Gas6–/– mice (P < 0.001).
Gas6 suppresses intestinal tumorigenesis

ApcMin AOM/DSS colitis mouse, the significantly severe anemia and shorter survival. In contrast with the ApcMin mice, the activities of NF-κB, a downstream target of TLR signal-
ing, and TNF-α mRNA levels were significantly higher in the Gas6–/– study, the activities of NF-κB signal-
ing, and TNF-α mRNA levels were significantly higher in the absence of Gas6. Although not directly, these in vivo results suggest that the predominant target of Gas6 was stromal infiltrating cells rather than epithelial cells, and that Gas6 inhibits intestinal inflammation in DSS-induced colitis by suppressing immune responses of stromal monocyte lineages (e.g. macrophages). These results also support the notion that the weak growth-promoting effect of Gas6 on epithelial cells may be overcome by strong immune suppressive effect of Gas6 on monocyte lineages during in vivo intestinal tumorigenesis.

It should be noted that similar to the AOM/DSS colitis mice, in the ApcMin mouse model in this study, the number of polyps in the Gas6−/− mice was greater than that in the Gas6+/+ mice, thereby resulting in significantly severe anemia and shorter survival. In contrast with the AOM/DSS colitis mouse, the ApcMin mouse is not characterized by the presence of obvious intestinal inflammation. However, polyps from both AOM/DSS and ApcMin mice are exposed to LPS-producing luminal bacteria. Lack of TLR signaling or microbes promotes ApcMin mouse intestinal tumorigenesis (3,4). Furthermore, lack of TNF-α, a key inflammatory cytokine that induces NF-κB activation, significantly reduces intestinal polyp formation in ApcMin mice (25). In this study, the activities of NF-κB, a downstream target of TLR signaling, and TNF-α mRNA levels were significantly higher in the Gas6−/− mice than in the Gas6+/+ mice with either AOM/DSS treatment or ApcMin genotype. Consistent with these data, Cox2 (Pigs2), a key tumor-promoting factor regulated by NF-κB, was also significantly elevated in the Gas6−/− mice. Therefore, activation of the TLR/TNF-α–NF-κB–Cox2 pathways appears to be one of the important events that promote intestinal tumorigenesis in the absence of Gas6 in both AOM/DSS-treated and ApcMin mouse models.

In humans, we found that higher expression of Gas6 in the cancer tissues was associated with longer survival and a milder local inflammatory response in colorectal cancer patients. These data are consistent with our murine models and with a previous study showing that high Gas6 expression was associated with better prognosis of renal cancer patients (18). However, a recent report showed that the activation of Axl is associated with the resistance to epidermal growth factor receptor-targeted therapy in lung cancer (16). In addition, it is important to note that warfarin, which has an inhibitory effect on Gas6 activity by blocking vitamin K-dependent γ-glutamyl carboxylation, is widely used as an anticoagulant. As for the association between warfarin use and the prognosis of cancer patients, a recent large cohort study showed that the effect of warfarin on patient survival was identical to that of placebo in gastrointestinal cancers (28). Thus, the role of Gas6 and related factors appears complex, and further studies are required to determine the comprehensive role of Gas6 and TAM receptors in colorectal cancer patients.

In conclusion, we demonstrated that Gas6 has an inhibitory effect on intestinal tumorigenesis in vivo. Although additional clinical studies are required to clarify the roles of Gas6 in human colorectal cancers, our results may provide clues for developing novel therapeutic strategies against colorectal cancer by regulating stromal immune responses.

Supplementary material
Supplementary Table 1–3 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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