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ORIGINAL RESEARCH

AIM2 regulates viability and apoptosis in human colorectal cancer cells via the PI3K/Akt pathway

Jianjun Chen
Zhenjun Wang
Sanshui Yu

Department of General Surgery, Beijing Chaoyang Hospital, Capital Medical University, Beijing, People's Republic of China

Abstract: Absent in melanoma 2 (AIM2) plays an important role in innate immunity as a DNA sensor in the cytoplasm by triggering the assembly of an AIM2 inflammasome that results in caspase-1-mediated inflammatory responses and cell death. In recent years, studies have indicated that AIM2 can suppress cancer cell proliferation, and mutations in the gene encoding AIM2 are frequently identified in patients with colorectal cancer (CRC). However, the mechanism by which AIM2 restricts tumor growth remains unclear. We reconstructed AIM2 expression in HCT116 CRC cells by lentivirus transfection. Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, we demonstrated that expression of AIM2 inhibited the viability and increased the apoptosis rate of CRC cells, and cell cycle analysis suggested that AIM2 blocked cell cycle transition from G1 to S phase. Western blot analysis showed that AIM2 promoted apoptosis in CRC cells by suppressing the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) pathway. Our data suggest that AIM2 plays a critical role as a tumor suppressor and might serve as a potential therapeutic target in CRC.

Keywords: AIM2, colorectal cancer, PI3K/Akt pathway, apoptosis

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death, and it is ranked as the second most common cancer in males and the third most common cancer in females worldwide according to Cancer Statistics for 2015.[1] Recent data have shown that the incidence of CRC in China has rapidly increased.[2] CRC is a heterogeneous disease with regard to molecular pathogenesis and clinical course, which makes it difficult to select patients who will benefit from therapies. Further study is required to identify new therapeutic targets in CRC.

Absent in melanoma 2 (AIM2) was originally reported as a tumor suppressor gene in melanoma in 1997 by DeYoung et al.[3] AIM2 is a member of the interferon-inducible PYRIN and HIN domain-containing (PYHIN) family proteins (also referred to as p200 family proteins).[4–9] Recently, AIM2 was found to act as a DNA sensor in innate immunity. By direct binding to foreign double-stranded DNA in infected macrophages, AIM2 triggers the assembly of an AIM2 inflammasome, resulting in caspase-1-mediated inflammatory responses and cell death, and thus contributes to the host defense against bacterial and viral pathogens.[10–15] Although the role of AIM2 in inflammasome activation is well accepted, its role in tumorigenesis is less clear. Exogenous AIM2 expression was shown to reduce human breast cancer cell proliferation by inhibition of nuclear factor kappa-B (NF-κB) transcriptional activity and to suppress mammary tumor growth in a mouse model.[16] A previous study showed that >50% of tumors from patients with small bowel cancer have frameshift mutations in the gene encoding

Correspondence: Zhenjun Wang
Department of General Surgery, Beijing Chaoyang Hospital, Capital Medical University, 8 Gongtinan Road, Chaoyang District, Beijing 100020, People's Republic of China
Tel +86 10 8523 1604
Email drzhenjun@163.com

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AIM2.[17] A better understanding of the biological function of AIM2 in CRC is key to identifying therapies that could be used in the treatment of this disease.

Mutations in the gene encoding AIM2 are frequently identified in patients with CRC.[18,19] Analysis of 414 colorectal tumors and matching control tissues further revealed that 67% of the tumors showed reduced expression of the gene encoding AIM2 relative to control tissues.[20] In this study, we aimed to identify the role of AIM2 expression in CRC cells and its underlying mechanism.

Materials and methods
Cell culture and lentivirus transfection
The human CRC HCT116 cell line was purchased from Genechem Co. (Shanghai, China) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2. Lentiviral vector containing AIM2 was purchased from Genechem Co. HCT116 cells were infected with lentivirus to generate cell lines with stable expression of AIM2. HCT116 cells were incubated with infection medium containing recombinant lentivirus vectors at a multiplicity of infection of 20 for 16 hours, and then the medium was replaced with fresh complete medium. Green fluorescent protein (GFP) expression was observed by fluorescence microscopy to determine the proportion of GFP-positive cells at 72 hours. Empty vector was used as a negative control. The transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting.

RNA isolation and qRT-PCR
Total RNA was extracted from HCT116 cells using TRI Reagent (Pufei International Trade Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Expression of target genes was detected by qRT-PCR using a QuantiFast SYBR Green PCR Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. Primers for the *AIM2* gene were as follows: forward 5′-CAGGAGGAGAAGGAGAAAGTTG-3′; reverse 5′-GTGCAGCACGTTGCTTTG-3′. Reactions of qRT-PCR were performed using MX3000p (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. A melting-curve analysis was performed to ensure specificity of the products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as an internal control. The relative mRNA levels of target genes were obtained by using the $2^{-\Delta\Delta Ct}$ method with all assays performed in triplicate.

The program calculates the ΔΔCT value with the following formulas: ΔΔCt = ΔCt mean (AIM2 expression group) − ΔCt mean (negative control group); fold change of gene expression $= 2^{-\Delta\Delta Ct}$.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
The cell viability was measured using the MTT assay. Twenty-four hours after transfection, cells were seeded in 96-well plates at a density of 2,000 cells per well for 24, 48, 72, 96, and 120 hours. To determine cell proliferation, cells were incubated with 20 μL of 5 mg/mL MTT for 4 hours at 37°C and then 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the crystals with 20-minute incubation at room temperature. Optical density was measured at a wavelength of 490 nm. All experiments were performed three times, and average results were used to generate the growth curves.

Flow cytometry
For cell cycle analysis, 5×10^6 AIM2-expressing cells and negative control cells were harvested at log phase and fixed in 70% ethanol for 2 hours at 4°C. Cells were washed three times with cold phosphate-buffered saline (PBS) and then stained with 50 μg/mL propidium iodide (PI) and 100 μg/mL ribonuclease for 30 minutes each at 4°C in the dark. The proportion of cells in each phase of the cell cycle was analyzed using the Modft LT2.0 DNA assay (Becton Dickinson). Each experiment was performed in triplicate.

For the cell apoptosis assay, cells were harvested including nonadherent cells and washed in PBS, and 5×10^6 cells from each group were stained with Annexin V-Allophycocyanin using the Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer's protocol. The apoptosis rate was determined by flow cytometric analysis using CellQuest software. Each experiment was performed in triplicate.

Phosphatidylinositol 3-kinase (PI3K) activation
To investigate the role of the PI3K/Akt pathway in the suppressive effect of AIM2 on regulating the viability and apoptosis of CRC cells, we treated cells with insulin-like growth factor-1 (IGF-1), which activates the PI3K/Akt pathway and is upregulated in colon cancer.[21] AIM2-overexpressing HCT116 cells and negative control cells were plated overnight and then stimulated with 100 ng/mL IGF-1 (ab87177; Abcam). After incubation for another 24 hours, the apoptosis rate was analyzed by flow cytometric analysis, and the level of p-Akt was assessed by Western blotting.

Western blotting

For Western blotting analysis, cells in culture were lysed using radioimmunoprecipitation assay (RIPA) buffer and the protein concentration was measured by bicinchoninic acid assay. Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk powder at room temperature, followed by the addition of primary antibodies against Akt1 (ab81283; Abcam) and GAPDH (sc-32233; Santa Cruz; as a control reference) and incubation overnight at 4°C on a shaking table. The membrane was washed three times, and then incubated with secondary antibodies for 1.5 hours at room temperature. Protein expression was detected using the enhanced chemiluminescence (ECL) substrate kit (Thermo Fisher Scientific, Rockford, IL, USA), and band intensity was quantified using ImageJ software.

Data analysis

Results were analyzed statistically using Student's *t*-test for comparisons between two groups. Correlation parameters were subjected to Pearson and nonparametric Spearman correlations. $P < 0.05$ was considered statistically significant.

Results

### Establishment and characterization of AIM2-expressing HCT116 CRC cells

Previous reports have demonstrated that overexpression of AIM2 inhibits the progression of CRC. We reconstituted the expression of AIM2 in human HCT116 CRC cells, which lack functional AIM2,[19] by transfection with AIM2-bearing lentiviral vector. The transfection efficiency was evaluated by qRT-PCR. As shown in Figure 1A, the level of *AIM2* mRNA was upregulated significantly (~7.7-fold; ΔΔCt =−2.95) by transfection with lentivirus-AIM2 compared with the negative control cells. Western blot analysis confirmed the expression of AIM2 in transfected cells (Figure 1B), and the quantification showed that the relative protein level of AIM2 compared with β-actin was significantly higher in the AIM2-expressing cells compared with control cells ($P < 0.01$, n=3; Figure 1C). These results confirmed the successful construction of recombinant lentivirus containing the human AIM2 gene that was capable of efficiently infecting HCT116 cells and upregulating AIM2 protein expression.

Effects of AIM2 on CRC viability and apoptosis

To determine the effect of AIM2 on cell viability, the MTT assay was used to evaluate the viability of AIM2-HCT116 and negative control cells. The growth curves (Figure 2A) showed that, relative to control cells, the viability of AIM2-HCT116 cells decreased significantly from day 1 to day 5 after transfection (n=3, $P < 0.01$); the cell growth inhibition rates for days 1–5 were 22.84%, 33.43%, 26.15%, 27.30%, and 27.62%, respectively.

The cellular apoptosis rate in AIM2-HCT116 and negative control cells was 4.76%±0.17% vs 3.46%±0.24%, respectively (Figure 2B); thus, AIM2-HCT116 cells
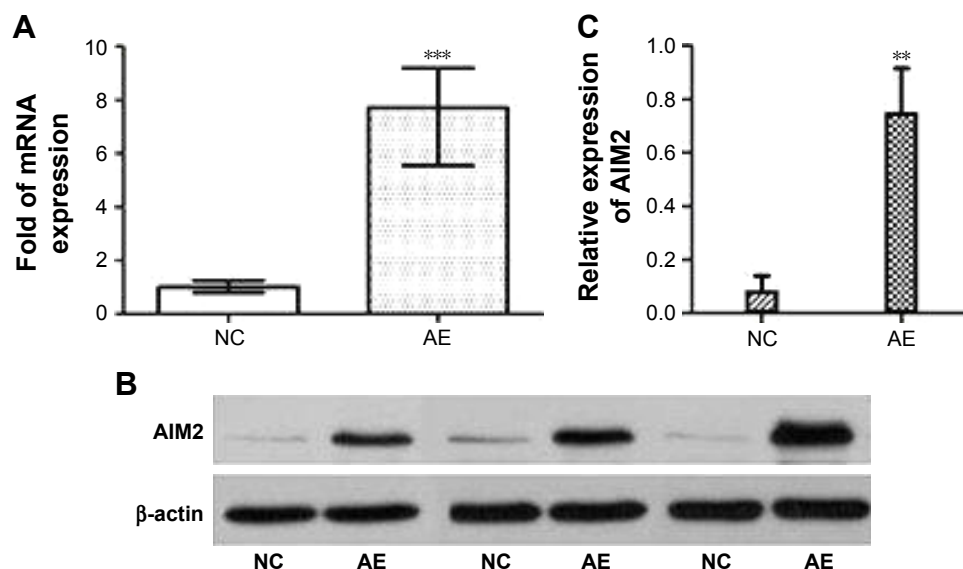
Figure 1 Expression of AIM2 mRNA and protein measured by RT-PCR and Western blotting.
Notes: (**A**) The expression of *AIM2* mRNA dramatically increased after lentivirus-AIM2 transfection. (**B**) Western blotting analysis of AIM2 protein expression in NC and AE cells. (**C**) Relative expression of AIM2 was significantly upregulated in the AE group. **$P < 0.01$, ***$P < 0.001$.
Abbreviations: AE, AIM2-expressing group; AIM2, absent in melanoma 2; NC, negative control; RT-PCR, real-time polymerase chain reaction.
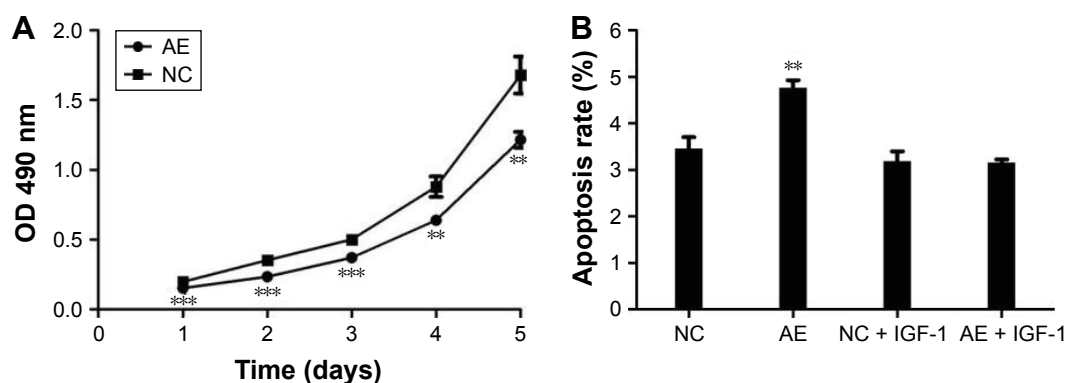
Figure 2 The effect of AIM2 on viability and apoptosis of HCT116 cells.
Notes: (**A**) MTT assay showed that the expression of AIM2 suppressed the cell viability compared with the negative control. (**B**) Cell apoptosis detected by Annexin V-APC dual-label flow cytometry. The apoptosis rate was significantly increased in the AE group; IGF-1 decreased the apoptosis rate of the AE group but had no significant effect in the NC group. **P<0.01; ***P<0.001.
Abbreviations: AE, AIM2-expressing group; AIM2, absent in melanoma 2; APC, Allophycocyanin; IGF-1, insulin-like growth factor-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control; OD, optical density.

exhibited a significantly higher rate of apoptosis (n=3, $P<0.01$).

Effect of AIM2 on cell cycle progression of CRC cells

To further investigate the influence of AIM2 on the proliferative ability of HCT116 CRC cells, the distribution of cells in different phases of the cell cycle was analyzed by flow cytometry. As shown in Figure 3, in AIM2-HCT116 CRC cells, the proportion of cells in G1 phase (45.56±0.51 vs 43.03±0.62, $P<0.01$) and G2/M phase (46.56±1.02 vs 44.04±1.11, $P<0.05$) was increased, whereas the proportion in S phase was decreased (7.88±1.1585 vs 12.93±0.94, $P<0.01$) relative to control-transfected cells.

Relationship between AIM2 and the PI3K/Akt pathway

Although the abovementioned data suggest that AIM2 regulates the viability and apoptosis of CRC cells, the underlying mechanism remained unclear. Therefore, we examined the nature of the intracellular signaling induced by AIM2 in CRC cells. Specifically, we examined the PI3K–Akt pathway, which modulates cell cycle progression, apoptosis, and cell motility and is frequently mutated in human CRC.[22]

Without IGF-1, AIM2-HCT116 cells presented a higher apoptosis rate than the negative control cells, whereas after treatment with IGF-1 the apoptosis rate of AIM2-HCT116 cells was not significantly different from that of the negative control (Figure 2B). These data suggest that Akt activation inhibits the capacity of AIM2 to induce apoptosis in CRC cells.

Results of Western blotting analysis showed that Akt activation (p-Akt) was nearly the same in AIM2-expressing

HCT116 cells and in empty vector-transfected control cells. Upon activation with IGF-1, the level of p-Akt was significantly lower in AIM2-HCT116 cells compared with negative controls (Figure 4), indicating that the expression of AIM2 resulted in significant suppression of Akt activation.

Overall, these data suggest that Akt activation (via phosphorylation) plays an important role in the inhibition of CRC progression by AIM2 and the proapoptotic effect of AIM2 is mediated via the inhibition of the PI3K/Akt pathway.

Discussion

Members of the pattern recognition receptor family are central players in the regulation of infections and inflammatory and metabolic diseases. AIM2 was recently discovered as a new member of this protein family. Studies have identified AIM2 as an innate immune DNA sensor that maintains intestinal homeostasis and prevents colitis and CRC, implicating a role as a tumor suppressor.[23,24] Two recent studies comparing azoxymethane/dextran sodium sulfate-induced colitis-associated colon cancer development in AIM2-deficient B6 mice or wild-type mice concluded that AIM2 expression protects against colitis-associated colon cancer in mice.[25,26] However, the mechanism of this effect remained unclear and controversial. The ability of AIM2 to tightly suppress overt cellular proliferation of intestinal epithelial cells may be the mechanism driving AIM2-dependent protection against tumorigenesis.

Our study showed that the AIM2 inhibited the viability and increased the apoptosis rate of CRC cells, in good agreement with reports that AIM2 inhibits the proliferation of a variety of human cancer cell lines, such as breast cancer cells, and murine fibroblast cells in vitro.[16,27–29] We demonstrate that AIM2-mediated inhibition of cell proliferation is associated

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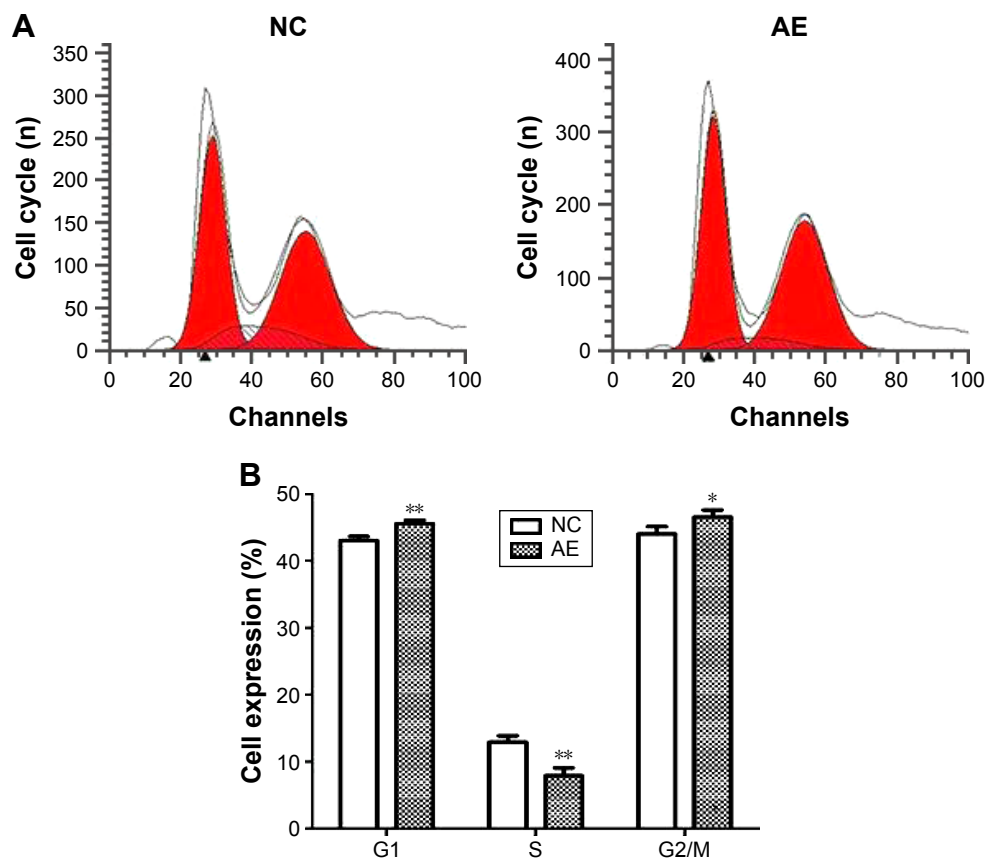
Figure 3 Cell cycle distribution of HCT116 CRC cells.
Notes: (**A**) NC and AE cells were fixed in ethanol, stained with PI, and analyzed by flow cytometry. (**B**) Expression of AIM2 increased the proportion of cells in S phase and decreased the proportion of cells in G1 and G2/M phases compared to control (n=3, *$P<0.05$, **$P<0.01$).
Abbreviations: AE, AIM2-expressing group; AIM2, absent in melanoma 2; CRC, colorectal cancer; NC, negative control; PI, propidium iodide.

with a decreased number of cells in S phase, indicating that expression of AIM2 may block cell cycle transition from G1 to S phase. Previous studies have demonstrated that the interferon-induced protein p202, which also belongs to the p200 family of proteins, has the ability to delay cell growth and retain cells in the G0/G1 phase.[30] PI3K/Akt signaling is important for many cellular activities including cell growth, survival, proliferation, and motility.[31–34] Upon activation of the PI3K/Akt pathway, the apoptosis rate of AIM2-expressing cells was the same as that of non-expressing cells, indicating
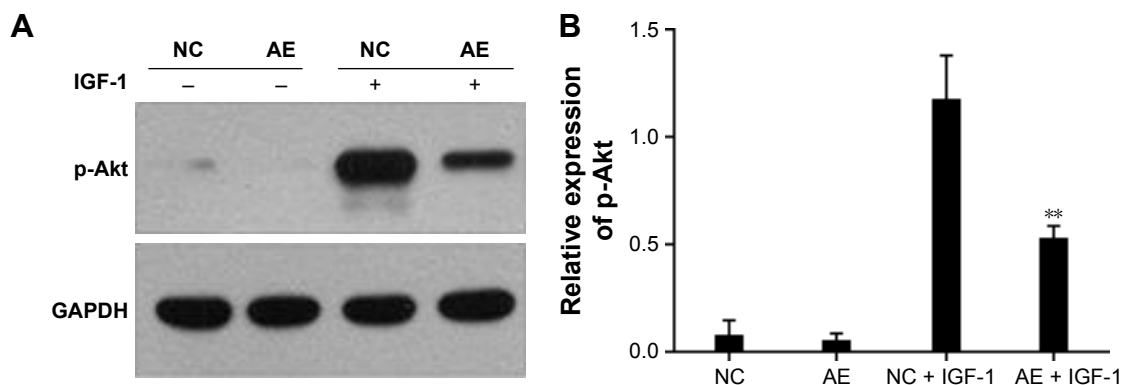
Figure 4 Effect of AIM2 expression on activation of the PI3K/Akt pathway in HCT116 cells.
Notes: (**A**) Western blot analysis of p-Akt protein level in NC and AE cells with and without IGF-1 treatment. (**B**) Quantification of the immunoreactivity showed no significant difference in the expression of p-Akt between NC and AE cells in the absence of IGF-1 (P=0.54), whereas after stimulation with IGF-1 the level of p-Akt was significantly lower in AE cells compared with NC cells (**$P<0.01$).
Abbreviations: AE, AIM2-expressing group; AIM2, absent in melanoma 2; Akt, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-1, insulin-like growth factor-1; NC, negative control; PI3K, phosphatidylinositol 3-kinase.

that activation of PI3K/Akt can suppress the proapoptotic effect of AIM2 in CRC cells. Moreover, Western blotting analysis demonstrated that expression of AIM2 suppressed the activation of PI3K/Akt. It can be concluded that AIM2 promotes apoptosis in CRC cells by suppressing the PI3K/Akt pathway. Hu et al[35] also studied the effect of AIM2 on the PI3K/Akt pathway and found that AIM2 protects intestinal integrity against *Salmonella* mucosal infection via Akt.

Conclusion

Our results show a potential antitumorigenic role for AIM2 in CRC that is likely mediated by inhibiting the PI3K/Akt pathway. This suggests that AIM2 plays a critical role as a tumor suppressor and might serve as a potential therapeutic target for future development of AIM2-based gene therapy for human CRC. Considering the complex nature of tumor growth and the biological diversity of CRCs, AIM2, like previously identified single markers, may be included in a set of multiple markers representing a characteristic expression profile of certain cancer subtypes. It will be important to discover whether different combinations of these marker sets result in more precise identification of patients who will benefit from specific therapies.

Disclosure

The authors report no conflicts of interest in this work.

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