Human Cancer Xenografts in Outbred Nude Mice can be Confounded by Polymorphisms in a Modifier of Tumorigenesis

Maged Zeineldin\textsuperscript{\textdaggerdash,\textasteriskcentered}, Derek Jensen\textsuperscript{\textdagger}, Smita R. Paranjape\textsuperscript{\textdagger}, Nikhil K. Parelkar\textsuperscript{\textdagger}, Iman Jokar\textsuperscript{\dagger}, George A. Vielhauer\textsuperscript{\textdagger}, and Kristi L. Neufeld\textsuperscript{\textdagger}

\textsuperscript{\textdagger}Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045, United States
\textsuperscript{\textasteriskcentered}Department of Human Genetics, Medical Research Institute, Alexandria University, Alexandria, Egypt
\textsuperscript{\dagger}Department of Urology, University of Kansas Medical Center, Kansas City, Kansas 66160, United States

genbank accession #s KF564039, KF564040, KF564041, KF564042, KF564043, KF564044, KF564045, KF564046, KF564047, KF564048, KF564049, and KF564050

Running title: Pla2g2a in Apc\textsuperscript{\textasciitilde}\textsuperscript{1322T} mice and nude mice

Key words: Pla2g2a/Mom1, Apc-mouse models, Nude mice, Xenograft model, Nonsense-mediated RNA decay

*Corresponding author: Kristi L. Neufeld, 7049 Haworth Hall, 1200 Sunnyside Ave., University of Kansas, Lawrence, KS 66045; phone (785)864-5079; fax (785)864-5294; email: klnuef@ku.edu

Copyright 2014.
ABSTRACT

Tumorigenicity studies often employ outbred nude mice, in the absence of direct evidence that this mixed genetic background will negatively impact experimental outcome. Here we show that outbred nude mice carry two different alleles of *Pla2g2a*, a genetic modifier of intestinal tumorigenesis in mice. We identified previously unreported linked polymorphisms in the promoter, noncoding and coding sequences of *Pla2g2a* and show that outbred nude mice from different commercial providers are heterogeneous for this polymorphic *Pla2g2a* allele. This heterogeneity even extends to mice obtained from a single commercial provider which display mixed *Pla2g2a* genotypes. Notably, we demonstrated that the polymorphic *Pla2g2a* allele affects orthotopic xenograft establishment of human colon cancer cells in outbred nude mice. This finding establishes a non-cell autonomous role for *Pla2g2a* in suppressing intestinal tumorigenesis. Using *in-vitro* reporter assays and pharmacological inhibitors, we show promoter polymorphisms and nonsense-mediated RNA decay (NMD) as underlying mechanisms that lead to low *Pla2g2a* mRNA levels in tumor-sensitive mice. Together, this study provides mechanistic insight regarding *Pla2g2a* polymorphisms and demonstrates a non-cell autonomous role for *Pla2g2a* in suppressing tumors. Moreover, our direct demonstration that mixed genetic backgrounds of outbred nude mice can significantly impact baseline tumorigenicity cautions against future use of outbred mice for tumor xenograft studies.
INTRODUCTION

Athymic nude mice, since their first use for tumorigenicity studies in the late 1960s (RYGAARD and POVLSEN 1969), have served as a model in over 40,000 publications. This model is used to assess the potential of tumor cells to proliferate, invade and metastasize, as well as the efficacy of anti-cancer therapeutics (SINGH and FERRARA 2012; ZHANG et al. 2012). Nude mice are defective in T cell-mediated immunity and therefore, are less prone to reject human cancer cell xenografts (RYGAARD and POVLSEN 1969). Although originally maintained as an inbred BALB/c mouse strain, an outbred line of nude mice with increased fertility and vigor is now the mainstay for xenograft studies. It has been suggested that the genetic variation found in outbred mice better recapitulates the heterogeneity found in the natural population. While true, this same genetic variation also confounds experiments performed using small samples of outbred mice.

Genetic modifiers are powerful tools to elucidate protein and pathway interactions that influence a phenotype. On C57Bl/6 (B6) genetic background, mice that harbor a germline truncating mutation in tumor suppressor Adenomatous polyposis coli (Apc^Min) develop from 20 to >100 intestinal tumors (polyps) and die around 20-weeks of age (MOSER et al. 1992; ZEINELDIN and NEUFELD 2013b; ZEINELDIN and NEUFELD 2013a). When comparing polyp numbers in Apc^Min mice in different strains, an even greater variation (0.5 - >300) is observed (HALBERG et al. 2009). Some of this variation could be attributed to environmental factors. In addition, 18 genetic modifiers were found to contribute to these wide variations in intestinal tumor number (KWONG and DOVE 2009; ZEINELDIN and NEUFELD 2013b; ZEINELDIN and NEUFELD 2013a). For some of these modifiers, specific sequence variants have been defined (KWONG et al. 2007; MCCART et al. 2008; OIKARINEN et al. 2009; CRIST et al. 2011; NNADI et al. 2012).
The first identified and best characterized genetic modifier of $Apc^{Min}$, Mom-1 was linked to a region of chromosome 4 containing the $Pla2g2a$ gene which encodes a secreted phospholipase A2 enzyme (Moser et al. 1992; Dietrich et al. 1993). $Apc^{Min}$ mice develop fewer intestinal polyps if they are from strains that show high intestinal expression of $Pla2g2a$ (tumor-resistant) and more intestinal polyps in mouse strains that show no detectable $Pla2g2a$ gene expression (tumor-sensitive) (MacPhee et al. 1995). Overexpression of $Pla2g2a$ in a transgenic mouse reduces the number of $Apc^{Min}$–induced polyps in the sensitive B6 reference strain, indicating that $Pla2g2a$ contributes to the Mom-1 phenotype (Cormier et al. 1997). Sequencing $Pla2g2a$ cDNA from multiple mouse strains revealed an extra base in exon 3 in tumor-sensitive but not in tumor-resistant strains (Kennedy et al. 1995; MacPhee et al. 1995). This frameshift mutation results in a premature stop and was associated with an alternatively spliced RNA (Kennedy et al. 1995; MacPhee et al. 1995). Because this nonsense mutation in tumor-resistant strains occurs before the last exon it was predicted to lead to nonsense mediated RNA decay (NMD) (MacPhee et al. 1995). NMD is a conserved eukaryotic mechanism to reduce the burden of mutant proteins by degrading mRNAs with premature stop codons (Palacios 2013). However, NMD of the $Pla2g2a$ transcript has not been demonstrated experimentally. In addition, whether $Pla2g2a$ reduces intestinal tumorigenesis in a cell autonomous or non-cell autonomous manner is not completely understood. As nude mice are maintained as outbred colonies the question remained whether they vary at $Pla2g2a$ locus and whether a $Pla2g2a$ genotypic variation can affect tumorigenesis in human xenograft studies using nude mice.

Here we identify new polymorphisms in the promoter, exons and introns of the $Pla2g2a$ gene, and confirm the previously identified exon 3 polymorphism in $Pla2g2a$. We show that these linked polymorphisms are present in tumor-resistant mouse strains ($Pla2g2a^{R}$) and associate with higher levels of intestinal $Pla2g2a$ mRNA. Commercially available outbred nude mice are heterogeneous for the $Pla2g2a$ polymorphisms, both when comparing mice from
different providers and among mice from a single provider. Importantly, nude mice harboring the
Pla2g2a<sup>R</sup> allele showed decreased establishment of human colon cancer cell xenografts. Finally, we also present evidence that both promoter polymorphisms and NMD participate in the mechanism underlying reduced Pla2g2a expression in tumor-sensitive strains.

**MATERIALS AND METHODS**

**Mice**

The research using mice in this study was conducted in accordance of OLAW and AAALAC guidelines and was approved by the University of Kansas and the University of Kansas Medical Center Institutional Animal Care and Use Committees. We received two Apc<sup>1322T</sup> male mice as a generous gift from Dr. Ian Tomlinson, Oxford University, UK. Apc<sup>Mn</sup> mice were purchased from Jackson laboratory (Bar-Harbor, ME). Both Apc<sup>1322T</sup> and Apc<sup>Mn</sup> mice were maintained in the University of Kansas Animal Care Unit by breeding males to wild-type C57Bl/6J females (Jackson laboratory, Bar-Harbor ME). Mice were genotyped according to the published protocols (POLLARD et al. 2009).

**Screening for Pla2g2a alleles**

The mouse strain that was used as reference for the UCSC genome sequence database is C57Bl/6 (http://genome.ucsc.edu/cgi-bin/hgGateway). We refer to this reference Pla2g2a sequence as the “B6 allele”. We refer to a Pla2g2a sequence that varies from that found in C57BL/6 mice as “polymorphic”. Genomic DNA was purchased directly from Jackson laboratory, provided as gifts from researchers at The University of Kansas, or prepared from tail tissue or fecal pellets. 1-2 mm of the end of the mouse tail was incubated in 200-µl buffer (50mM KCl, 50mM Tris HCl (pH 8.0), 2.5 mM EDTA, 0.45% NP-40 and 0.45% Tween-20) containing 100 µg proteinase-K at 55 °C until tissue was completely digested. Proteinase-K was
inactivated by heating at 95 °C for 5 minutes. For DNA isolation from fecal pellets (figure 3B), each fecal pellet was collected in a 1.5-ml tube containing 1-ml phosphate buffered saline (PBS). The sample was vortexed for 2 minutes at the maximum speed followed by centrifugation at 700Xg for 5 minutes. 180-µl of the supernatant was taken to a new tube and 20-µl proteinase-K (20mg/ml) was added. Proteinase-K digestion for 2 hours at 55 °C was followed by enzyme inactivation at 95 °C for 5 minutes. DNA was further purified using DNeasy blood and tissue kit [Qiagen] following manufacturer’s instructions.

To distinguish the polymorphic from the B6 Pla2g2a allele, we designed a primer that is complementary to the polymorphic sequence and is different from the B6 sequence in its last two 3’ nucleotides (figure 1B). This primer also has a mismatch mutation at the invariable nucleotide at position -4 from the 3’ end. Introducing this mismatch reduces primer annealing to the B6 Pla2g2a DNA at its 3’ region, preventing non-specific amplification without interfering with annealing of the same primer to the polymorphic allele DNA. We used the same strategy to design a primer specific for the B6 allele that does not amplify the polymorphic allele (Table 1 shows primers sequences). PCR was performed in a 25 µl reaction containing 2 µl genomic DNA (5 µl for DNA isolated from fecal pellets), 2 mM MgCl₂, 0.4 mM dNTPs (NEB), 15 picomoles of the common primer (Pla2ga2F), 3 picomoles of the Pla2g2a⁸R primer, and 10 picomoles of the Pla2g2a⁹R primer (Table 1 shows primers sequences) and 1 unit of Crimson Taq DNA polymerase (NEB). The reaction conditions were 94 °C for 5 minutes, then 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds and extension at 68 °C for 30 seconds followed by a final extension at 68 °C for 3 minutes. Pla2g2a alleles were separated by electrophoresis in 2% agarose gels and detected by ethidium bromide staining and UV visualization.

**Sequencing the Pla2g2a gene and regions of Mom-2, Mom-5, & Mom-7**

The expected lifespans of Apc<sup>Min</sup> and Apc<sup>1322T</sup> mice are 20 and 16 weeks, respectively (Moser et al. 1990; Pollard et al. 2009). Initial analysis of long-lived mice (all C57Bl/6 strain)
included one $Apc^{Min}$ mouse that lived for 57 weeks and one $Apc^{1322T}$ mouse that lived for 37 weeks. These mice were compared to an $Apc^{Min}$ mouse that lived for 18 weeks and another $Apc^{1322T}$ mouse that became sick and was sacrificed at 18 weeks as short-lived controls. We also included two wild-type C57Bl/6J mice purchased directly from the commercial provider (Jackson Laboratories, Bar Harbor, ME).

Genomic DNA was amplified with overlapping primers covering the entire $Pla2g2a$ gene, including promoter, 5'UTR, exons, introns and 3'UTR (Table 1). For analysis of $Mom2$, $Mom5$ and $Mom7$, sequence variation-specific primers spanning published sequences were used to amplify DNA by PCR (Table 1) (BARAN et al. 2007; KWONG et al. 2007; OIKARINEN et al. 2009). This examination did not lead to identification of a candidate modifier. Gel-purified PCR products were sent for Sanger sequencing (ACGT Inc. or Genewiz). Sequences were compared to the C57Bl/6 mouse reference sequences at the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The complete $Pla2g2a$ sequence from different strains has been uploaded in genbank (accession #'s: KF564039, KF564040, KF564041, KF564042, KF564043, KF564044, KF564045, KF564046, KF564047, KF564048, KF564049, and KF564050).

Analysis of mRNA by real-time reverse transcription–PCR

Intestinal epithelial cells were isolated from 3 mice with B6 $Pla2g2a$ and 3 mice with polymorphic $Pla2g2a$ alleles as described (ZEINELDIN and NEUFELD 2012). RNA isolation and cDNA formation from intestinal epithelial cells, HCT116 cells or cecum of the nude mice was performed as previously described (ZEINELDIN et al. 2012). Briefly, 1-ml of Trizol® (Invitrogen) was added to 200-µl of suspended intestinal epithelial cells. For cultured HCT116 cells, cell media was removed and cells were then scraped in 1-ml of Trizol® (Invitrogen) and moved into 1.5-ml tubes. Samples were stored at -80°C until use. RNA isolation and purification using Trizol® (Invitrogen) was performed according to manufacturer’s instructions. For nude mouse cecal tissue, the cecum was opened, washed in PBS, a small piece (~3 mm) was placed into a
1.5 ml tube and snap-frozen in dry ice and kept at -80°C until use. Samples were homogenized in 1-ml Trizol® (Invitrogen) using a hand-held homogenizer. RNA was isolated from 350 µl of the homogenized lysate using Direct-Zol RNA miniprep (Zymo reseach) following manufacturer’s protocol. The quality of the isolated RNA was assessed using 1% agarose gel electrophoresis and quantity was determined by measuring the OD$_{260}$. For preparing cDNA, 1 µg total RNA was incubated for 1 h at 42°C in 1X M-MLuV enzyme buffer (NEB, Ipswich, MA, USA) containing 1mM dNTPs, 1 µg random hexamer primers (NEB), and 200U of M-MLuV reverse transcriptase enzyme (NEB). The reverse transcriptase enzyme was then inactivated by heating at 95°C for 5 min. Quantitative PCR was performed for mouse Pla2g2a, mouse Hprt, bacterial Amp and Human HPRT using specific primers listed in Table 1 and using a DNA engine Opticon 2 instrument (MJ Research, Waltham, MA, USA). The total reaction volume was 25 µl, containing 1X DyNAmo HS SYBR Green qPCR kit (Thermo), 15 pmol of each primer and 2 µl of cDNA at a 1:5 dilution. Each reaction was performed in duplicate or triplicate. The reaction steps were initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. Fluorescence was measured at the end of every cycle and a melting curve was analyzed between 40°C and 95°C, with 0.2°C increments. Samples were included in the analysis only if the melting curve was a single peak at the expected temperature. For quantifying Pla2g2a mRNA in intestinal epithelial cells and ceca of nude mice, the average ΔC(t) was calculated for every sample relative to the housekeeping gene transcript Hprt. In calculating fold-change of Pla2g2a mRNA from intestinal epithelial cells, ΔΔC(t) method was used relative to the C57Bl/6 mice with B6 Pla2g2a sequence. For quantification of Pla2g2a transcript in the ceca from nude mice, the relative abundance for every sample was normalized to the median value for resistant nude mice that did not establish tumors. In quantifying mouse Pla2g2a transcript in HCT116 cells, ΔC(t) was calculated relative to Amp-resistant sequence within the transfected plasmid. To correct for any variation in
transfection efficiency between samples, Amp abundance was normalized to human HPRT expression and GAPDH DNA sequences. P-values were calculated using the two-tailed Mann–Whitney non-parametric test and GraphPad Prism software.

**Cloning and plasmids**

To test the effect of promoter polymorphisms on Pla2g2a expression, Pla2g2a-Prom(AKR):pGL3 and Pla2g2a-Prom(C58):pGL3 reporter plasmids were made by inserting Pla2g2a promoter (a 1.5 Kb DNA piece upstream to Pla2g2a first codon) and 5'UTR DNA from AKR or C58 mouse strains upstream of the Luciferase reporter gene in the pGL3 promoter vector using “Pla2g2a Prom” and “Pla2g2a 5'UTR” primers (listed in Table 1). For testing Pla2g2a mRNA stability, all Pla2g2a coding exons and intervening introns from C58 and AKR mouse strains were used to replace the Luciferase gene in the pGL3 promoter vector using “Pla2g2a whole” primers (listed in Table 1). All the cloned plasmids were sequenced at ATCG and only those with no cloning-induced mutations were included in the study.

**Luciferase reporter assay**

Using GeneExpresso Transfection Reagent following manufacturer’s instructions, 2.25 µg of either Pla2g2a-Prom(AKR):pGL3 promoter or Pla2g2a-Prom(C58):pGL3 promoter was co-transfected with 0.25 µg of Renilla Luciferase (transfection control) into HCT116 cells. After 48 hours, cells were harvested and luciferase activity was assessed using a Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a LMAXII 384 microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase data were normalized to Renilla luciferase. Fold change of the normalized firefly luciferase from the Pla2g2a-Prom(AKR):pGL3 promoter was calculated relative to the normalized firefly luciferase from the Pla2g2a-Prom(C58):pGL3 promoter. Data were collected from five independent experiments. P-values were calculated using the Mann–Whitney non-parametric test and GraphPad Prism software.

**Testing Pla2g2a mRNA stability and NMD**
To test Pla2g2a mRNA stability, HCT116 cells were transfected with 2.5 µg of Pla2g2a(AKR):pGL3 or Pla2g2a(C58):pGL3 plasmid using GeneExpresso Transfection Reagent following manufacturer’s instructions. After 24 hours, RNA was isolated and mouse Pla2g2a transcripts were quantified by RT-PCR using mouse-specific Pla2g2a primers. Data were collected from four independent experiments including 11 samples transfected with Pla2g2a(AKR):pGL3 and 10 samples transfected with Pla2g2a(C58):pGL3 DNA. To test NMD, transfected cells were treated with DMSO vehicle or 20 µM wortmannin for 2 hours, 100 nM cyclohexamide for 6 hours, or both for 6 hours before quantifying mouse Pla2g2a mRNA levels. Data were collected from three independent experiments.

**Nude mice xenograft experiment**

Thirty five 6-8 week-old female Nu/Nu mice (provider B) were orthotopically inoculated with HCT116 cells expressing m-cherry/Luciferase. Implantation of HCT116 cells and imaging of mice were performed by investigators blind to the mouse genotypes. Briefly, mice were anesthetized with pentobarbital (50 mg/kg) and the cecum was accessed via a small incision into the peritoneal wall. With the aid of a dissecting microscope, 1 x 10⁶ cells were injected in a volume of 60 µl into the submucosal layer of the cecum. Following survival surgery and while animals were recovering, a tail snip was collected from each animal to allow for genotyping as described above. The mice were allowed to recover and then returned to housing. The study continued for 28 days from the date of cell implantation and animals were imaged once weekly using bioluminescence imaging (described below) to quantify tumor burden.

**Bioluminescence imaging**

Animals were injected with potassium salt of D-Luciferin (150 mg/kg body weight). Following isoflurane-induced anesthesia, animals were imaged at 15 min after D-Luciferin injection using a Xenogen IVIS system coupled to Living Image acquisition and analysis software version 4.0 (PerkinElmer, Waltham, MA). Region of interest (ROI) boxes were drawn around the entire body (excluding tail) of the animals or around the tissue specimen for ex-vivo
imaging. Measurements were expressed as total flux i.e. photons/second (p/s).

RESULTS

**Novel Pla2g2a polymorphisms identified**

Genetic modifiers can alter tumorigenicity in mouse cancer models. One such modifier, \(\text{Pla2g2a}\) has long been known to affect tumor formation in mice genetically predisposed to intestinal tumorigenesis, \(\text{Apc}^{\text{Min}}\). Sequence analysis of the entire \(\text{Pla2g2a}\) gene including promoter, 5'UTR, exons and introns revealed previously unknown sequence differences in a heterozygous state in a long-lived \(\text{Apc}^{\text{Min}}\) mouse from our colony, but not in \(\text{Apc}^{\text{Min}}\) mice with more typical short lifespans or in wild-type C57Bl/6J mice obtained directly from a commercial provider (figure 1A). We refer to this sequence variation as “polymorphic” when compared to the C57BL/6 reference sequence [UCSC genomic sequence database] which will be referred to as “B6”. Many of these polymorphisms were found in the promoter region of \(\text{Pla2g2a}\) however some were also found in other coding and non-coding regions of the gene. The long lived \(\text{Apc}^{\text{Min}}\) mouse did not have alterations in other described genetic “modifiers of Min” (\(\text{Mom2, Mom5 and Mom7}\)) as determined by direct sequencing of PCR amplified genomic DNA (BARAN et al. 2007; KWONG et al. 2007; OIKARINEN et al. 2009).

To screen for mice with \(\text{Pla2g2a}\) polymorphisms, we developed a simple test that could accurately differentiate between the B6 allele and the polymorphic allele in a single PCR reaction. Using a common forward oligomer/primer that anneals to \(\text{Pla2g2a}\) promoter DNA in both the B6 and polymorphic alleles and two specific reverse primers that recognize either the B6 or polymorphic allele (figure 1B), two PCR products (272- and 353-bp) are amplified.
Polymorphisms found throughout the \textit{Pla2g2a} gene in different mouse strains

To unambiguously determine the sequences of the polymorphic loci, we amplified the promoter regions from each \textit{Pla2g2a} allele obtained from a heterozygous mouse, introduced this DNA into a plasmid, and sequenced these plasmid DNAs. We found 26 bases that differed between the B6 and the polymorphic alleles in the \textit{Pla2g2a} promoter, 5'UTR region and first intron. The sites of these polymorphisms relative to the transcriptional start site are; -882, -879, -874, -869, -865, -857, -851, -832, -804, -801, -692, -689, -529, -517, -479, -359, -348, -347, +48, +319, +361, +382, +479, +599, +648 and +711. As these \textit{Pla2g2a} polymorphisms were not previously reported, we screened additional mouse strains using our PCR-based screening protocol and found that seven of the eleven congenic strains also contained the polymorphisms (figure 1C). Sequencing the \textit{Pla2g2a} promoter region and 5'UTR DNA from these strains confirmed the PCR results (figure 1D, 1E). Of note, we found that the polymorphic \textit{Pla2g2a} allele was present in strains that were previously shown to be tumor-resistant; AKR, DBA/1, DBA/2, BALB/c and C3H/He (DIETRICH \textit{et al.} 1993; MACPHEE \textit{et al.} 1995; HALBERG \textit{et al.} 2009).

We tested whether mice with \textit{Pla2g2a} promoter polymorphisms identified in our colony would also carry a previously identified \textit{Pla2g2a} exon 3 alteration (absence of the “T” insertion found in the reference sequence from B6 mice) (KENNEDY \textit{et al.} 1995). Sequencing exon 3 in five mice that were heterozygous for \textit{Pla2g2a} promoter polymorphisms as shown by PCR screening and promoter sequencing (figure 1C and 1D), revealed that all were heterozygous for the exon 3 “T” nucleotide alteration as well (not shown). In contrast, 5 mice that were homozygous for the B6 \textit{Pla2g2a} promoter sequence were also homozygous for the corresponding B6 sequence alteration in exon 3 (a “T” nucleotide insertion). We conclude that the \textit{Pla2g2a} allele detected in our colony and referred to as “polymorphic” compared to the reference B6, also has the “T” alteration in exon 3 that was previously reported. In addition to this alteration, we found many other “linked” polymorphisms in coding and non-coding
sequences that associated with tumor-sensitive or tumor-resistant strains. These polymorphisms include a missense mutation 76 bp upstream from the original exon 3 insertion that is predicted to result in a tryptophan to arginine substitution in all tumor-sensitive strains (figure 1F) and a similar missense mutation 21 bp downstream only in AKR and DBA/1 strains. Polymorphisms were also detected in other intron and coding exons. Figure 1G shows a polymorphic locus in exon 4. The complete Pla2g2a sequence from different strains has been uploaded in genbank (accession numbers provided in Materials and Methods). These linked polymorphisms indicate extensive Pla2g2a sequence variation between sensitive and resistant mouse strains.

**Pla2g2a Polymorphisms in outbred nude mice**

Unlike congenic inbred mouse strains which are homozygous for virtually all chromosomal loci, outbred mice maintain genetic variation in individual mice. Using our PCR-based test, we found two out of the five outbred CD1 mice analyzed were heterozygous for the Pla2g2a polymorphic allele (Pla2g2a\(^R\)) while the other three mice were homozygous for the B6 allele (Pla2g2a\(^S\)), consistent with a previous report of the Pla2g2a exon 3 base insertion in CD1 mice (figure 2A) (KENNEDY et al. 1995). We confirmed these results by sequencing Pla2g2a from one mouse of each genotype and show the promoter sequence from a CD1 mouse in figure 1D.

Because most nude mice are maintained as outbred colonies, genetic variation is preserved among these mice. Using our PCR protocol to screen genomic DNA isolated from 103 nude mice (Nu/Nu) from 2 different providers, we found all 57 mice from provider ‘A” were homozygous for the Pla2g2a allele carried by resistant strains (Pla2g2a\(^R\), figure 2B). In contrast, nude mice from provider “B” displayed various combinations of both alleles (Pla2g2a\(^S\) and Pla2g2a\(^R\)). Out of 46 mice from provider “B”, 10 (21.7%) were Pla2g2a\(^S\)\(^S\), 28 (60.9%) were
*Pla2g2a*<sup>S</sup> and *Pla2g2a*<sup>R</sup> allele in these mice was 0.543 and 0.457, respectively. DNA sequencing of the *Pla2g2a* promoter region and exon 3 from four *Pla2g2a*<sup>S/S</sup>, four *Pla2g2a*<sup>R/R</sup> and three *Pla2g2a*<sup>R/S</sup> nude mice confirmed the presence of the “B6” (*Pla2g2a*<sup>S</sup>) or “polymorphic” (*Pla2g2a*<sup>R</sup>) alleles, including the previously reported exon 3 insertion of “T” nucleotide, as predicted by PCR screening results, again indicating that these polymorphisms are linked (data not shown).

**Pla2g2a affects orthotopic tumor establishment of colon cancer cells in nude mice, functioning in a non-cell autonomous manner**

Whether *Pla2g2a* suppression of tumorigenesis is cell-autonomous or non-cell autonomous is not settled. We reasoned that, as nude mice were heterogeneous for the polymorphic *Pla2g2a* allele, orthotopic injection of colon cancer cells in the cecum would allow us to directly test a cell autonomous versus a non-cell autonomous role for *Pla2g2a* in intestinal tumor suppression. In addition, this system would also allow us to test our hypothesis that *Pla2g2a* heterogeneity can affect the outcome of xenograft studies where human cancer cells are injected into nude mice and then tumor development and growth is monitored. To test these hypotheses, we purchased 35 female nude mice from provider “B” and injected luciferase-labeled HCT116 human colon cancer cells into the cecal wall of these mice. Xenograft establishment, growth and local and distant metastasis were monitored (figure 3A). Because the *Pla2g2a*<sup>R</sup> allele appears to be dominant (Dietrich et al. 1993; Cormier et al. 1997), we combined 5 *Pla2g2a*<sup>R/R</sup> and 22 *Pla2g2a*<sup>S/R</sup> mice into a single “resistant” group (R) with the remaining 8 *Pla2g2a*<sup>S/S</sup> mice forming the “sensitive” group (S). HCT116 cells established cecal tumors in 75% of the sensitive group mice but only 34.6% of the resistant group mice (Chi squared = 4.047, two tailed p = 0.0443, figure 3C). Over the four week duration of the experiment, four out of the six tumors (67%) that developed in the sensitive mice grew bigger while only 44.4% of the tumors established in resistant mice expanded (Table 2). At the end of
the experiment, two tumors (33%) from mice in the sensitive group demonstrated invasion, one local, and one more distant metastasis to the liver (figure 3B and Table 2). In the resistant group, only one tumor had local metastasis (11.1%). These results demonstrate a non-cell autonomous role for Pla2g2a in suppression of colon tumors. Of potentially broader impact, this analysis also provides a concrete example of how a genetic modifier, can significantly alter the outcome of a xenograft study performed using outbred nude mice.

Mice with Pla2g2a polymorphisms have more Pla2g2a mRNA in intestinal cells

The Pla2g2a allele in C57Bl/6 mice (Pla2g2a<sup>S</sup>) has been described as virtually null (MACPHEE et al. 1995). However, the reduction in Pla2g2a expression was attributed to the exon 3 “T” insertion. Since we found previously unreported polymorphisms in Pla2g2a, with many of them in the promoter region, we were interested to measure the effects of these polymorphisms on Pla2g2a expression. We first compared Pla2g2a expression in intestinal epithelial cells from our C57BL6 mice with known Pla2g2a genotypes. We found that mice with a polymorphic allele (Pla2g2a<sup>R</sup>) had higher Pla2g2a mRNA levels in epithelial cells from jejunum, ileum, and colon (177-, 31- and 77-fold by qRT-PCR, respectively, data not shown) than mice with only the B6 allele (Pla2g2a<sup>S</sup>). Figure 3E shows RT-PCR products from jejunal RNA isolated from mice with different Pla2g2a alleles amplified with Pla2g2a- or Hprt-specific primers. Therefore, the Pla2g2a polymorphisms we initially identified in long-lived mice associate with increased expression of the gene.

Reduced Pla2g2a mRNA level also correlates with tumor sensitivity in Apc<sup>Min</sup> mice (MACPHEE et al. 1995). To examine the link between establishment of orthotopic xenograft tumors and Pla2g2a expression, we performed quantitative RT-PCR using mouse-specific Pla2g2a primers and RNA isolated from ceca of nude mice. We observed wide variation in Pla2g2a mRNA levels in the cecum from nude mice, especially among the resistant group. This
variation may represent heterogeneous genetic elements controlling $Pla2g2a$ expression in these mice with mixed genetic background. Nonetheless, mice carrying the $Pla2g2a^R$ allele showed higher levels of $Pla2g2a$ mRNA than the sensitive group mice (figure 3D, $p=0.003$). Within the sensitive group, the mice that didn’t establish tumors had significantly more $Pla2g2a$ mRNA than those that established tumors (figure 3D). The same trend of higher $Pla2g2a$ mRNA level in mice that didn’t establish tumors was also seen in the resistant group, although this did not reach statistical significance.

**$Pla2g2a$ polymorphisms associate with prolonged survival of $Apc^{1322T}$ mice**

It was critical to identify all mice in our colony that had $Pla2g2a$ polymorphisms, so that they could be excluded from future studies. Using the PCR-based test to screen our mice, we found the $Pla2g2a$ polymorphic allele in only two $Apc^{Min}$ mice, but in many mice with another germline $Apc$ mutation, $Apc^{1322T}$ (figure 4A). As most $Apc^{1322T}$ mice with the polymorphic allele were euthanized at different time points and many of these mice underwent different experimental conditions, compiling their survival or intestinal tumor data was not informative. However, we found that 5 $Apc^{1322T}$ mice with the B6 $Pla2g2a$ allele and 7 $Apc^{1322T}$ mice heterozygous for the polymorphic allele died or were sacrificed when they were moribund, thus providing useful survival data. While $Apc^{1322T}$ with the B6 $Pla2g2a$ gene lived for 16.4 ± 1.9 weeks, which is comparable to the reported survival of $Apc^{1322T}$ mice (Pollard et al. 2009; Lewis et al. 2010), $Apc^{1322T}$ mice heterozygous for the polymorphic allele lived significantly longer, until 26.3 ± 2.3 weeks ($p= 0.0075$) (figure. 4B). Together, these data indicate that the $Pla2g2a$ polymorphic allele associates with prolonged survival in $Apc^{1322T}$ mice.

To trace the source of the $Pla2g2a$ polymorphic allele in our $Apc^{Min}$ and $Apc^{1322T}$ colonies, we screened the available DNA from many generations of mice. We conclude that the
polymorphic Pla2g2a allele was introduced into our Apc\textsuperscript{Min} and Apc\textsuperscript{1322T} mice by a breeding female carrying the polymorphic allele obtained from another provider.

**Promoter polymorphisms affect Pla2g2a expression**

To understand the mechanisms underlying the low expression of the “B6” Pla2g2a allele, we initially examined the newly identified Pla2g2a promoter polymorphisms for their contribution to gene expression. Using several programs to predict differences in transcription factor binding between the B6 and polymorphic Pla2g2a alleles we did not identify a consistent set of transcription factors (data not shown). Nonetheless, to look for global effects of the promoter polymorphisms, we generated firefly luciferase reporter constructs driven by the Pla2g2a promoter and 5’UTR regions, including the first intron, from the AKR strain (Pla2g2a\textsuperscript{R}) or C58 strain (Pla2g2a\textsuperscript{S} allele identical to that of C57Bl/6 mice) (figure 5A). We found significantly more firefly luciferase activity in cells transfected with the AKR-derived Pla2g2a promoter and 5’UTR reporter than in cells transfected with the C58-derived Pla2g2a promoter and 5’UTR (figure 5A). We conclude that the Pla2g2a promoter polymorphisms affect Pla2g2a expression, resulting in Pla2g2a RNA levels that are higher in tumor-resistant strains than in tumor-sensitive strains.

**NMD contributes to instability of Pla2g2a transcript in tumor-resistant strains**

It was previously proposed that low levels of Pla2g2a mRNA in tumor-sensitive mice were the result of nonsense mediated RNA decay (NMD) (MacPhee \textit{et al.} 1995). This assumption, based on the known polymorphism in Pla2g2a positioned in exon 3 and thus before the last exon, has not been directly tested. To examine the potential involvement of NMD in controlling Pla2g2a levels, a second set of constructs was generated by cloning the Pla2g2a coding exons and the intervening introns from tumor-sensitive (C58, which carries an allele identical to that of C57Bl/6 mice) or tumor-resistant (AKR) strains after the SV40 promoter. We reasoned that as the Pla2g2a gene from each strain was controlled by the same strong
promoter, any changes in transcript level would likely result from inherent RNA stability differences rather than transcription rates. Using qRT-PCR and primers specific for mouse Pla2g2a mRNA, we observed that Pla2g2a mRNA levels are significantly lower in human cells transfected with the construct containing the C58-derived Pla2g2a sequence than in those transfected with the AKR-derived Pla2g2a sequence (figure 5B).

To further confirm a role for NMD in reduction of Pla2g2a mRNA levels in tumor-sensitive strains, we treated cultured cells with pharmacologic NMD inhibitors. Wortmannin is a PI3K inhibitor that can also inhibit the PI3K-related protein hSMG1, a component of the NMD surveillance machinery (PAL et al. 2001). Cyclohexamide inhibits eukaryotic protein synthesis and thereby also affects NMD which requires protein synthesis for completion (PALACIOS 2013). When human cells transfected with the SV40-driven C58-derived Pla2g2a gene construct were treated with wortmannin alone or in combination with cyclohexamide, the mouse Pla2g2a mRNA level significantly increased relative to vehicle-treated cells (figure 5C). In contrast, a parallel control experiment performed using the SV40-driven AKR-derived Pla2g2a gene construct revealed no change in mouse Pla2g2a transcript level in the cells treated with wortmannin, cyclohexamide or both drugs (figure 5C). We conclude that Pla2g2a promoter polymorphisms and NMD each contribute to Pla2g2a mRNA levels in tumor-sensitive strains.

DISCUSSION

Genetic and environmental factors modify tumorigenicity in humans and in model organisms such as mice. Identification and characterization of genetic modifiers leads to greater appreciation for the different pathways contributing to a given phenotype (KWONG and DOVE 2009). In this study, we showed that there are two distinct Pla2g2a alleles in different mouse strains: one allele is similar to that described in the reference sequence (B6 allele, Pla2g2aS)
and the other is polymorphic (Pla2g2a\textsuperscript{R}). The Pla2g2a\textsuperscript{R} allele is different from the B6 (Pla2g2a\textsuperscript{S}) allele in the promoter, coding and non-coding sequences. We first identified this Pla2g2a polymorphic allele in long-lived Apc\textsuperscript{Min} mice in our colony. Most of these polymorphisms are conserved in tumor-resistant mouse strains. Pla2g2a polymorphisms in outbred nude mice affect the orthotopic establishment of human cancer cells. In addition, we provide evidence that promoter polymorphisms affect Pla2g2a transcription and that Pla2g2a transcripts from tumor-sensitive mice undergo NMD.

Outbred mice have genetic variation that is lacking in inbred congenic mouse lines. But this same feature that provides more natural population characteristics, may also confound experimental results, particularly in studies using only a small sample size (CHIA et al. 2005). One of the most unexpected results from the current study was the finding that commercially available outbred nude mice are heterogeneous for Pla2g2a alleles and this heterogeneity leads to variation in the capacity of each outbred mouse to support growth of a human cancer cell xenograft (figure 2 and 3). Nude mice from two different commercial providers were not consistent in Pla2g2a genotype. While all nude mice from one provider were homozygous for the Pla2g2a\textsuperscript{R} allele, both the Pla2g2a\textsuperscript{R} and Pla2g2a\textsuperscript{S} alleles were present in the mice from a second provider. These findings highlight the potential danger of comparing the results from studies performed using nude mice from different providers and even results from a heterogeneous mix of outbred nude mice from a single provider. For instance, nude mice in our study were shipped in seven cages with five mice per cage. As expected, there was variable distribution of Pla2g2a alleles between cages, with the frequency of Pla2g2a\textsuperscript{S/S} mice in each cage ranging from 0/5 (0%) to 3/5 (60%). Results from this study unambiguously demonstrate the necessity of a large sample size in studies using outbred nude mice.

The heterogeneity of outbred nude mice is further illustrated by the varied Pla2g2a transcript levels, even in nude mice with the same Pla2g2a genotype (figure 3D). This variation
presumably results from additional differences in genetic elements that regulate gene expression. It seems likely, but remains to be tested whether the related Pla2g2a transcript level will affect orthotopic xenografts of other colon cancer cells and other tumorigenesis studies with different cancer cells and injection sites. Nonetheless, the inability to completely monitor or control for varied expression of tumorigenesis modifiers such as Pla2g2a makes tumorigenicity studies using outbred mice problematic.

This is the first report of a genetic modifier of \( Apc^{Min} \) that also affects the phenotype of \( Apc^{1322T} \) mice. The \( Apc^{1322T} \) model was developed to more closely recapitulate the longer truncated APC proteins (\(~\) half full-length) found in human colorectal cancers (Nieuwenhuis and Vassen 2007; Pollard et al. 2009). In this study we showed that \( Pla2g2a^R \) prolongs the survival of \( Apc^{1322T} \) mice. Mom-1 was originally described as a quantitative trait locus that affects polyp multiplicity, size and distribution in \( Apc^{Min} \) mice. Although survival is a good indicator for polyp multiplicity, we looked retrograde at collected polyp data from our \( Apc^{1322T} \) mice. Unfortunately, most of these mice carrying the \( Pla2g2a^R \) allele were sacrificed at different time points and were under different experimental conditions making this analysis uninformative. A future study of \( Apc^{1322T} \) mice with different \( Pla2g2a \) genotypes and sacrificed at the same age would be necessary to detect any effect of Pla2g2a on polyp multiplicity, size and distribution.

Another Apc model, the \( Apc^{\Delta 242} \) mouse, with a much shorter APC protein truncation, showed fewer polyps in the first generation of C3H/He X C57Bl/6 mice (Crist et al. 2010). Notably, the C3H/He mouse strain has the tumor-resistant \( Pla2g2a \) allele (figure 1C) (Markova et al. 2005). Taken together, our results in \( Apc^{1322T} \) mice and reported data in \( Apc^{Min} \) and \( Apc^{\Delta 242} \) mice (Dietrich et al. 1993; Crist et al. 2010) indicate that \( Pla2g2a \) polymorphisms can affect intestinal tumorigenesis in Apc-mutant mice, independent of the nature of Apc mutation.
Although Pla2g2a is responsible for most of the Mom-1 phenotype in Apc\textsuperscript{Min} mice, the Pla2g2a gene is only one part of the Mom-1 locus. Another locus distal to Pla2g2a, but still within the Mom-1 locus, has a modest effect on polyp multiplicity in small intestines of Apc\textsuperscript{Min} mice (CORMIER et al. 2000). In the present study, we examined only Pla2g2a polymorphisms in Apc\textsuperscript{1322T} and nude mice and not the distal Mom-1 locus. Perhaps the distal Mom-1 locus accounts for some of the prolonged survival phenotype in Apc\textsuperscript{1322T} mice. The distal Mom-1 locus might also contribute to the variation in xenograft establishment in nude mice ceca. However the previous finding that Pla2g2a and not the distal Mom-1 locus is responsible for all the resistance phenotype in the colon (CORMIER et al. 2000), argues against this possibility.

Pla2g2a does not appear to function as a universal tumor suppressor or oncogene. PLA2G2A is upregulated in lung and prostate cancer cells (OLEKSIOWICZ et al. 2012; YU et al. 2012a). Furthermore, reducing expression of PLA2G2A by hairpin RNA reduces the proliferation of lung cancer cells, consistent with an oncogenic role for PLA2G2A (YU et al. 2012b). On the other hand, low PLA2G2A expression in gastric tumors is associated with increased invasiveness and metastasis, with poor prognostic outcome (GANESAN et al. 2008). Additionally, mouse strains with little or no expression of Pla2g2a have increased susceptibility to intestinal tumorigenicity driven by germline Apc mutations (DIETRICH et al. 1993; CRIST et al. 2010), Muc2 mutations (FIJNEMAN et al. 2008) or inflammation (FIJNEMAN et al. 2009). Here we show that orthotopic establishment of colon cancer cells is also reduced with higher Pla2g2a expression in nude mice. These data clearly establish a protective effect for Pla2g2a against intestinal tumorigenesis in mice.

The mechanism by which the phospholipase 2A enzyme Pla2g2a decreases intestinal tumor burden in resistant strains is not completely understood. Pla2g2a is expressed in Paneth cells in the small intestine and goblet cells in the large intestine. Secreted Pla2g2a may function in a non-cell autonomous manner by controlling bacterial flora and in so doing, reduce
inflammatory mediators that would otherwise promote tumor growth (FIJNEMAN and CORMIER 2008; FIJNEMAN et al. 2008). Pla2g2a can also modulate signaling pathways, including Wnt signaling, potentially through regulating production of fatty acids such as arachidonic acid, a precursor for a variety of prostaglandin signaling mediators (FIJNEMAN et al. 2009). Our orthotopic xenograft study clearly demonstrates a non-cell autonomous role for Pla2g2a. HCT116 human colon cancer cells express little endogenous Pla2g2a (BELINSKY et al. 2007), yet we found that their growth in the cecal wall of nude mice was affected in a manner that correlated with the level of cecal Pla2g2a transcript (figure 3D). The number of polyps that develop when fetal small intestinal tissues from ApcMin mice were grafted subcutaneously was previously shown to correlate with the Mom-1 allele status (sensitive or resistant) of the isograft rather than of the host mouse (GOULD and DOVE 1996). This result does not necessarily conflict with our cecal xenograft data, as Mom-1 could still affect intestinal tumorigencity in a non-cell autonomous manner within isograft’s intestinal tissue microenvironment in addition to a systemic effect in the host mouse.

It was previously reported that overexpression of mouse Pla2g2a in HCT116 cells increased xenograft tumor size when these colon cancer cells were implanted subcutaneously in nude mice (BELINSKY et al. 2007). This seemingly contradictory result might be explained by differences in the cell expressing Pla2g2a (implanted human cancer cells vs. surrounding mouse tissues) or the site of the xenograft implantation (subcutaneous vs. cecal wall). Moreover, in the previous study, the Pla2g2a genotype of the nude mice was not reported and this variable would be expected to affect the experimental outcome.

In conclusion, we showed that the Pla2g2a sequence varies between tumor-resistant and tumor-sensitive mouse strains. Some of these variations in the promoter region affect Pla2g2a expression and we provide direct evidence that NMD also contributes to reducing Pla2g2a mRNA level in tumor-sensitive strains. The Pla2g2a genotype affects not only the
Apc\textsuperscript{Mn} phenotype, but also that of Apc\textsuperscript{1322T} mice. Remarkably, the Pla2g2a genotype varies in outbred nude mice from different providers and this variation affects orthotopic establishment of colon cancer cell xenografts in these mice.

Acknowledgments
This work was supported by RO1 CA10922 and P30 CA168524 from the National Cancer Institute, P20 RR016475 from the National Center for Research Resources and P20 GM103418 from the National Institute of General Medical Sciences. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Dr. Ian Tomlinson, University of Oxford, UK for generously providing us with Apc\textsuperscript{1322T} mice. We would like also to thank Dr. Andrew L. Kung at the Dana Farber Cancer Institute for providing the FUW-Luc-mCherry-puro construct, and Drs. Liang Xu, Laird Forest, Cory Berkland, Mary Lou Michaelis, Charlotte Vines and Phillip Hardwidge from The University of Kansas and The University of Kansas Medical Center for providing mouse tissues for genotyping. We extend our gratitude to Vinit Nanavaty, Mahlet Yeshitla, William McGuinness, and Bryan Blanchat for technical support and to Staff at the Animal Care Unit at the University of Kansas for their assistance with mouse husbandry.

REFERENCES


Cormier, R. T., A. Bilger, A. J. Lillich, R. B. Halberg, K. H. Hong et al., 2000 The Mom1AKR intestinal tumor resistance region consists of Pla2g2a and a locus distal to D4Mit64. Oncogene 19: 3182-3192.


Fujineman, R. J., L. K. Bade, J. R. Peham, M. A. van de Wiel, V. W. van Hinsbergh et al., 2009 Pla2g2a attenuates colon tumorigenesis in azoxymethane-treated C57BL/6 mice; expression studies reveal Pla2g2a target genes and pathways. Cell Oncol 31: 345-356.

Fujineman, R. J., and R. T. Cormier, 2008 The roles of sPLA2-IIA (Pla2g2a) in cancer of the small and large intestine. Front Biosci 13: 4144-4174.


Lewis, A., S. Segiditas, M. Deheragoda, P. Pollard, R. Jeffery et al., 2010 Severe polyposis in Apc(1322T) mice is associated with submaximal Wnt signalling and increased expression of the stem cell marker Lgr5. Gut 59: 1680-1686.


Markova, M., R. A. Koratkar, K. A. Silverman, V. E. Sollars, M. MacPhee-Pellini et al., 2005 Diversity in secreted PLA2-IIA activity among inbred mouse strains that are resistant or susceptible to Apc Min/+ tumorigenesis. Oncogene 24: 6450-6458.


Nnadi, S., R. Watson, J. Innocent, G. Gonye, A. Buchberg et al., 2012 Identification of five novel modifier loci of Apc(Min) harbored in the BXH14 recombinant inbred strain. Carcinogenesis **33**: 1589-1597.


Oleksowicz, L., Y. Liu, R. Bracken, K. Gaïonde, B. Burke et al., 2012 Secretory phospholipase A2-IIa is a target gene of the HER/HER2-elicited pathway and a potential plasma biomarker for poor prognosis of prostate cancer. The Prostate **72**: 1140-1149.


Zeineldin, M., and K. Neufeld, 2012 Isolation of Epithelial Cells from Mouse Gastrointestinal Tract for Western Blot or RNA analysis pp. in Bio-protocol.


FIGURE LEGENDS

Figure 1: Pla2g2a polymorphisms identified in Apc<sub>Min</sub> mice. (A) Chromatograph showing Pla2g2a promoter sequence for a short-lived (B6) and a long-lived (Polymorphic) Apc-mutant mouse. Polymorphisms designated by red arrow. (B) PCR-based assay for Pla2g2a polymorphisms identified in promoter sequence of long-lived (Polymorphic) Apc-mutant mouse. (C) Screening different mouse strains for the Pla2g2a promoter polymorphisms using the PCR-based test. Strains AKR, DBA/2, DBA/1, C3H/He, BALB/c, FVB, and SJL carry the polymorphic promoter while strains C57, C58, A/J, P/J and 129/Sv carry the “B6” promoter. “Het”, heterozygous control sample from our mouse colony; “-”, PCR product without template; “M”, 100 bp DNA ladder. (D - G) Sequence results illustrate Pla2g2a polymorphisms found in different strains in the promoter area (D), first intron (E), exon 3 (F) and exon 4 (G). For exon 3 polymorphisms (E), sequence results illustrate the previously reported exon 3 nucleotide polymorphism marked by a black arrow; A newly identified R63W polymorphism in AKR and DBA/1 strains is marked by a blue arrow. Numbers represent nucleotide location, with +1 as the transcription start site.

Figure 2: Pla2g2a alleles in outbred mouse colonies. (A) PCR screening results from two CD1 mice are shown next to a control sample from a Pla2g2a<sup>R/S</sup> mouse (# 3). Mouse 1 is homozygous for the Pla2g2a<sup>S</sup> allele while mouse 2 is heterozygous (Pla2g2a<sup>R/S</sup>). (B) Screening nude mice from provider “A” using DNA from tail (upper panel) or stool (lower panel) revealed all mice to be homozygous for Pla2g2a<sup>R</sup>. (C) Screening nude mice from provider “B” revealed heterogeneity at the Pla2g2a locus. Mouse 1 & 3 are Pla2g2a<sup>R/S</sup>, mouse 2 is Pla2g2a<sup>S/S</sup> and mouse 4 & 5 are Pla2g2a<sup>R/R</sup>. As controls for panels B and CS, mouse 6, 7 and 8 are Pla2g2a<sup>S/S</sup>, Pla2g2a<sup>R/S</sup> and Pla2g2a<sup>R/R</sup>. “-”, PCR product without template. ”M”, 100 bp DNA ladder.
Figure 3: The \textit{Pla2g2a} allele decreases colon cancer cell establishment in the cecum of nude mice. (A) \textit{In vivo} image of luciferase-labeled HCT116 grafted in cecal wall of a sensitive (left panel) or resistant (right panel) nude mouse. (B) \textit{Ex-vivo} image of HCT116 luciferase-labeled cells showing hepatic metastasis (left), and local invasion (middle), both in sensitive nude mice, and local invasion (right) in a resistant nude mouse. (C) Percentage of sensitive and resistant nude mice in which HCT116 cells established tumors in the cecal wall. * indicates $p<0.05$ (Chi Square test). (D) Quantitative-RT-PCR revealed relative expression of \textit{Pla2g2a} RNA in the cecum of sensitive and resistant nude mice that established or did not establish tumors in colon cancer cell orthotopic xenografts. Line represents median. (E) RT-PCR of \textit{Pla2g2a} mRNA isolated from Jejunal intestinal epithelial cells of B6-\textit{Pla2g2a}^{S/S} mice or mice heterozygous for the polymorphic allele (Hetero- \textit{Pla2g2a}^{R/S}). The house keeping \textit{Hprt} was used as an internal control.

Figure 4: \textit{Pla2g2a} polymorphisms associate with prolonged survival of \textit{Apc}^{1322T} mice. (A) Analysis of genomic DNA from 10 \textit{Apc}^{1322T} mice. Samples 1, 3, 4, 5 and 6 are from mice heterozygous for the \textit{Pla2g2a} polymorphic allele, samples 2, 7, 8, 9 and 10 are from B6 mice. “-”, PCR product without template; ”M”, 100 bp DNA ladder. (B) The survival of \textit{Apc}^{1322T} mice with B6 \textit{Pla2g2a} status or heterozygous for the polymorphic \textit{Pla2g2a} allele. Lines indicate median survival for each group. * indicates $p < 0.05$ (two-tailed t-test).

Figure 5 Promoter polymorphisms and NMD alter \textit{Pla2g2a} RNA level (A) A luciferase reporter was expressed in HCT116 cells under the control of \textit{Pla2g2a} promoter DNA from the tumor-sensitive (S), C58 or the tumor-resistant (R), AKR strain. The \textit{Pla2g2a} promoter from the tumor-resistant strain resulted in more luciferase than that from the sensitive strain. * indicates $p< 0.05$ (Mann-Whitney non-parametric test). (B) qRT-PCR analysis of mouse \textit{Pla2g2a} mRNA levels from the tumor-sensitive strain (C58) and the tumor-resistant strain (AKR) \textit{Pla2g2a} genes each transcribed from the SV40 promoter in HCT116 cells. * indicates $p< 0.05$ (Mann-Whitney non-parametric test).
non-parametric test). (C) NMD was inhibited using 20 µM wortmannin (Wort.) and/or 100 nM cyclohexamide (Chx.). RNA levels were quantified by qRT-PCR. Note NMD inhibitors significantly increased C58 Pla2g2a mRNA level. * indicates $p < 0.05$ (Mann-Whitney non-parametric test).

### Table 1. PRIMERS USED IN THIS STUDY

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer</th>
<th>Backward primer</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mom1-1</td>
<td>5'-gtagtgctgccgtga-3'</td>
<td>5'-cataccttgcccttttg-3'</td>
<td>Promoter region</td>
</tr>
<tr>
<td>Mom1-5'UTR-1</td>
<td>5'-ccagtcggaggtttca-3'</td>
<td>5'-ggactcattccccagaattg-3'</td>
<td>5'UTR region</td>
</tr>
<tr>
<td>Mom1-5'UTR-2</td>
<td>5'-atcggctgcaagagaatg-3'</td>
<td>5'-ctgaaaccctggaact-3'</td>
<td>5'UTR region</td>
</tr>
<tr>
<td>Mom1-2</td>
<td>5'-ggcagttggaattcagga-3'</td>
<td>5'-tgagcttgaaaggaatgg-3'</td>
<td></td>
</tr>
<tr>
<td>Mom1-3</td>
<td>5'-tcacaccccctaccaggtc-3'</td>
<td>5'-acctggctgcttgaaac-3'</td>
<td></td>
</tr>
<tr>
<td>Mom1-4</td>
<td>5'-gttagcggcactccttc-3'</td>
<td>5'-atcctgggccacactgtc-3'</td>
<td></td>
</tr>
<tr>
<td>Mom1-5</td>
<td>5'-ccaaagggccacacaca-3'</td>
<td>5'-agttgctgaggtcacttg-3'</td>
<td></td>
</tr>
<tr>
<td>Mom1-6</td>
<td>5'-cccctgctgttatgaa-3'</td>
<td>5'tcagttgtagaacacacag-3'</td>
<td></td>
</tr>
<tr>
<td>Mom1-7</td>
<td>5'-aggccctcacaagtaagca-3'</td>
<td>5'-cctgtttttagtgctctc-3'</td>
<td>3'UTR region</td>
</tr>
<tr>
<td>Mom-2</td>
<td>5'-accatcctccagcacaag-3'</td>
<td>5'-ggcagatggacttaatgct-3'</td>
<td>Flanks exon 3 of Atp5a</td>
</tr>
<tr>
<td>Mom-5</td>
<td>5'-tgtggaagctgttttgga-3'</td>
<td>5'-aatgcatagatacctgtgag-3'</td>
<td></td>
</tr>
<tr>
<td>Mom-7</td>
<td>5'-aaccagctgctcctt-3'</td>
<td>5'- ttagaaccaggacagag-3'</td>
<td></td>
</tr>
</tbody>
</table>
### Primers for screening for *Pla2g2a* promoter polymorphisms

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’ direction)</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pla2g2aF</td>
<td>5’-tgattttgaaacctcctctga</td>
<td>Common primer</td>
</tr>
<tr>
<td>Pla2g2aR</td>
<td>5’-acttcacaaagctttcctgaac</td>
<td>Wild-type-specific primer</td>
</tr>
<tr>
<td>Pla2g2a’R</td>
<td>5’-tctgtatccctgaatgtctca</td>
<td>Polymorphism-specific primer</td>
</tr>
</tbody>
</table>

### Primers for cloning *Pla2g2a* promoter and 5’UTR regions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’ direction)</th>
<th>Sequence (5’-3’ direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pla2g2a Prom</td>
<td>5’-gcccggcttaccgtgtctgctctcccat</td>
<td>5’-gcggccctttcagttctgaattttcaactcc</td>
</tr>
<tr>
<td>Pla2g2a 5’UTR</td>
<td>5’-gcccggcccttaggaaacaagacaagcccctggaaacaa</td>
<td>5’-gcggccctgctagctcagctcagctgaaggacaa</td>
</tr>
</tbody>
</table>

### Primers for cloning *Pla2g2a* coding exons and intervening introns

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’ direction)</th>
<th>Sequence (5’-3’ direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pla2 W</td>
<td>5’-gcccggcccatggatgaaggtcctcctgcgctag-3’</td>
<td>5’-gcccggcttacgtagttctgaattttcaactcc</td>
</tr>
</tbody>
</table>

### Primers for QRT-PCR for *Pla2g2a* mRNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’ direction)</th>
<th>Sequence (5’-3’ direction)</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pla2g2a</td>
<td>5’-tacaagcgccctggagaaaag-3’</td>
<td>5’-gccccttacgcactgacaca-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse Hprt</td>
<td>5’-tgctcgagatgtcatgaagg-3’</td>
<td>5’-tactttccctggacgcctagttctgaattttcaactcc</td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>5’-ggtcctcctaggtctcagcgctaactatt-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human HPRT</td>
<td>5’-tgacactggccaaacaatgca-3’</td>
<td>5’-ggtctcttttcaccagcaagctactagttctgaattttcaactcc</td>
<td></td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>5’-ccatcactggccacccagaag-3’</td>
<td>5’-agctttcccgttcagctcagg-3’</td>
<td>DNA sequences</td>
</tr>
</tbody>
</table>
Table 2. ORTHOTOPIC TUMOR ESTABLISHMENT OF HUMAN COLON CANCER CELLS IN NUDE MICE

<table>
<thead>
<tr>
<th></th>
<th>Pla2g2a = Sensitive</th>
<th>Pla2g2a = Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>Mice with established tumors</td>
<td>6 (75%)</td>
<td>9 (34.6%)</td>
</tr>
<tr>
<td>Tumors growing</td>
<td>4 (67%)</td>
<td>4 (44.4%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>1 (16.7%)</td>
<td>0</td>
</tr>
<tr>
<td>Local invasion</td>
<td>1 (16.7%)</td>
<td>1 (11.1%)</td>
</tr>
</tbody>
</table>
Figure 4

(A) Gel electrophoresis showing the presence of the B6 (353 bp) and Polymorphic (272 bp) alleles.

(B) Survival analysis comparing B6 and Polymorphic promoter alleles. The asterisk indicates a statistically significant difference (*).