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Atorvastatin inhibits pancreatic carcinogenesis and increases survival in *LSL-Kras^{G12D}-LSL-Trp53^{R172H}-Pdx1-Cre* mice

Jie Liao*, Yeon Tae Chung, Allison L Yang, Meng Zhang, Haonan Li, Wanying Zhang, Liang Yan, and Guang-Yu Yang*

Department of Pathology, Northwestern University, Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, Illinois, 60611

Abstract

There are several studies supporting the role of HMG-CoA reductase inhibitors such as atorvastatin against carcinogenesis, in which inhibiting the generation of prenyl intermediates involved in protein prenylation plays the crucial role. Mutation of *Kras* gene is the most common genetic alteration in pancreatic cancer and the Ras protein requires prenylation for its membrane localization and activity. In the present study, the effectiveness of atorvastatin against pancreatic carcinogenesis and its effect on protein prenylation were determined using the *LSL-Kras^{G12D}-LSL-Trp53^{R172H}-Pdx1-Cre* mouse model (called *Pan^{Kras/p53}* mice). Five-week-old *Pan^{Kras/p53}* mice were fed either an AIN93M diet or a diet supplemented with 100 ppm atorvastatin. Kaplan-Meier survival analysis with Log-Rank test revealed a significant increase in survival in mice fed 100 ppm atorvastatin (171.9 ± 6.2 days) compared to the control mice (144.9 ± 8.4 days, $p < 0.05$). Histologic and immunohistochemical analysis showed that atorvastatin treatment resulted in a significant reduction in tumor volume and ki-67-labeled cell proliferation. Mechanistic studies on primary pancreatic tumors and the cultured murine pancreatic carcinoma cells revealed that atorvastatin inhibited prenylation in several key proteins, including Kras protein and its activities, and similar effect was observed in pancreatic carcinoma cells treated with farnesyltransferase inhibitor R115777. Microarray assay on the global gene expression profile demonstrated that a total of 132 genes were significantly modulated by atorvastatin; and Waf1p21, cyp51A1 and soluble epoxide hydrolase were crucial atorvastatin-targeted genes which involve in inflammation and carcinogenesis. This study indicates that atorvastatin has the potential to serve as a chemopreventive agent against pancreatic carcinogenesis.

Keywords

Atorvastatin; pancreatic adenocarcinoma; mouse model; chemoprevention; *LSL-Kras^{G12D}-LSL-Trp53^{R172H}-Pdx1-Cre* mice

Introduction

Statins have been widely used for the treatment of hypercholesterolemia and the prevention of coronary artery disease, and are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG CoA) reductase, the major rate-limiting enzyme in the mevalonate pathway of cholesterol synthesis. HMG-CoA reductase controls the conversion of HMG-CoA to mevalonic acid, which is the precursor for the biosynthesis of several fundamental

*To whom correspondence and reprint requests should be addressed: Guang-Yu Yang, M.D., Ph.D. Department of Pathology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611, Fax: 312-503-3419, g-yang@northwestern.edu Or Jie Liao, MD, Department of Pathology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611, Fax: 312-503-0645, jie-liao@northwestern.edu.

end-products including cholesterol, isoprenoids, dolichol, ubiquinone, and isopentenyladenine¹. All of these end-products play crucial roles in promoting carcinogenesis, particularly isoprenoids such as farnesal and geranylgeranyl which are key intermediate products involved in protein prenylation, a posttranslational lipid modification.^{2, 3} This lipid modification process exerts an effect on a wide range of signal transduction pathways including cell growth, differentiation, maintenance of the cellular cytoskeleton and vesicle trafficking through protein-protein interactions and anchoring to cellular membranes.²⁻⁵ Ras and Ras-related GTP-binding proteins (G proteins) require prenylation to enable cellular localization and function;⁴ and recent studies have demonstrated that the conversion of the protein-cysteine acceptor site in oncogenic Ras prevents prenylation and abolishes its malignant transforming ability.⁶ Thus, in addition to lowering cholesterol levels, targeting protein prenylation by statin drugs such as atorvastatin is crucial and possible mechanism for inhibiting oncogenic Kras activity and cancer.

Extensive experimental evidence indicates that statins have a chemopreventive potential against cancer and recent comprehensive reviews have also indicated that statins appear safe in relation to cancer risk.⁷⁻¹⁶ Although overall epidemiological studies of statin trials have been inconclusive in regards to cancer risk including pancreatic cancer, the safety and affordability of statins, the experimental evidence thus far, and the effect on reducing the risk of cardiovascular disease suggest that the study of statins on chemoprevention warrants further exploration to gain more experimental evidence, elucidate mechanism, and pursue future clinical trials on cancer.¹⁷

Animal model studies using statins have counteracted the actions of chemically-induced carcinogenesis in the colon, mammary gland, liver, and lung;¹⁸⁻²⁰ however, the role of HMG-CoA reductase inhibitors such as atorvastatin in targeting pancreatic carcinogenesis and Kras protein prenylation have not yet been studied. Multiple gene alterations have been identified in human pancreatic carcinogenesis. Activating point mutations in the *Kras* gene, the earliest events in pancreatic carcinogenesis,^{42, 43} have been shown to be present in approximately 95 percent of pancreatic cancers.^{21, 22, 23} Mutation of the tumor suppressor p53 is another common event. Great progress in developing mouse models of pancreatic cancer have been achieved using the genetic engineering approach to highly mimic the multiple genetic alterations involved in human pancreatic carcinogenesis²⁴. *LSL-Kras^{G12D}-LSL-Tip53^{R172}-Pdx-1-Cre* mice (called *Pan^{Kras/p53}* mice) have concomitant endogenous expression of transgenic mutant *Kras^{G12D}* and *p53^{R172}* in the pancreas and 100% of the mice develop pancreatic carcinomas²⁴. Histopathological analysis confirms that the pancreatic tumors were ductal adenocarcinomas, well to moderately-differentiated, with metastasis in the liver, lung and adrenals. Thus, *Pan^{Kras/p53}* mice are a most novel mouse model of pancreatic carcinogenesis, and highly mimicking to human pancreatic cancer from genetic alteration to morphology.

In the present study, we have determined the effectiveness of atorvastatin, a HMG-CoA reductase inhibitor, against pancreatic carcinogenesis in *Pan^{Kras/p53}* mice. Histopathologic and immunohistochemical analysis were further performed to evaluate pancreatic carcinogenesis, cell proliferation and mutant *Kras*-activated signals such as phosphorylated C-raf. Using pancreatic tumor tissues and *in vitro* cultured murine pancreatic carcinoma cell lines, the key prenylated proteins, including Kras, nuclear lamin A, and nuclear HDJ-2 protein (a co-chaperone of heat shock protein 70), were further determined using a western blot approach. Modulation of global transcriptional gene expression by atorvastatin was analyzed in the cultured murine pancreatic carcinoma cell lines using an Illumina gene array approach.

Materials and Methods

Animals

Six-week-old breeding pairs of genetically engineered mice, including transgenic *Pdx1-Cre*, *LSL-Trp53^{R172H}*, and *LSL-Kras^{G12D}* mice were used. *Pdx1-Cre* mice were kindly provided by Dr. Lowy (University of Cincinnati). *LSL-Trp53^{R172H}* and *LSL-Kras^{G12D}* mice were obtained from MMHCC, NCI/NIH and kindly provided by Dr. T Jacks (MIT). All of these genetically engineered mice were bred and genotyped in our lab following the protocols provided by the investigators²⁴. To produce compound transgenic *Pan^{kras/p53}* mice, the double transgenic *LSL-Kras^{G12D/+}-LSL-Trp53^{R172/+}* mice were first generated, and then further mated with heterozygous *Pdx1-Cre* transgenic mice. Mice were housed under pathogen-free conditions in the facilities of Laboratory Animal Services, Northwestern University. All studies were conducted in compliance with the Northwestern University IACUC guidelines.

Animal Experiments

To determine the effects of atorvastatin against pancreatic carcinogenesis, 4 weeks-old *Pan^{kras/p53}* mice were fed either an AIN-93M diet or an AIN-93M diet supplemented with 100 ppm atorvastatin throughout the experimental period. Selection of 100 ppm atorvastatin is based on the maximum tolerant dose, nutrient intensity²⁵, and the body surface area (BSA) normalization method²⁶, which is similar as an intermediate dosage used in human. Atorvastatin (as tablet form, 80 mg/tablet) was purchased from Pfizer Ireland Pharmaceuticals, Dublin, Ireland. The AIN-93M diet was purchased from Research Diets Inc., New Brunswick, NJ.

Animals were monitored daily for the development of pancreatic carcinoma, with the markers being cachexia, abdominal distension and weight loss >20%. Animals with these signs generally died within two days due to late stage pancreatic carcinoma, and thus were sacrificed immediately. All of animals were followed closely for the duration of survival from the initial development of pancreatic cancer. When the accumulated mortality reached statistically significant (in general, >75% in a group), all of the remaining animals were sacrificed.

Tissue Preparation and Histopathological Evaluation

After sacrificing the mice by CO₂ asphyxiation and cervical dislocation, pancreases and other key organs were collected. The number and size of the tumors were recorded, and three perpendicular diameter measurements were obtained for each tumor using calipers. Tumor volume was determined by the equation $V = 4/3\pi r^3$, where *r* was the average tumor radius obtained from the three diameter measurements. The pancreas and other organs were then fixed in 10% formalin for 24 h, routinely processed, and embedded in paraffin. Serial paraffin sections (5 μm) were made and stained with hematoxylin and eosin (H&E) for histopathological examination. Additional tissue sections were obtained on poly-L-lysine coated slides for immunohistochemical analyses.

Pancreatic tumors were analyzed histopathologically according to the established criteria²⁷. The tumors were classified as pancreatic ductal adenocarcinomas (PDAC), with or without sarcomatoid features based on the glandular architecture and spindle shape morphology of the PDAC. Chronic pancreatitis and precancerous lesions were also analyzed²⁷.

Immunohistochemistry

Immunohistochemical staining was performed according to our routine protocol using an avidin-biotin-peroxidase method²⁸. After slides were incubated with primary antibody (1

μg/ml for Ki-67 antibody or for CM-5p P53 antibody or 6μg/ml anti-myeloperoxidase antibody), the appropriate specific staining signals were amplified by incubation with a biotinylated secondary antibody and the avidin-biotin-peroxidase complex and highlighted by 3,3'-diaminobenzidine (the chromagen).. Anti-Ki-67 and anti-p53 antibodies were purchased from Vector Labs, CA. 3,3'-diaminobenzidine was used as the chromagen. Negative controls were established by replacing the primary antibody with PBS and normal serum. Positive staining was indicated by the presence of a brown-colored precipitate in the nucleus.

Cell culture, proliferation and colony formation assays

Three *in vitro* cultured mouse pancreatic carcinoma cell lines (P01 and P02 and M02) derived from either primary or metastatic carcinomas from *Pan^{kras/p53}* mice were well established and used for this study. Cell lines were allowed to grow in a monolayer. Cell proliferation and tumorigenicity were determined using a colony formation assay. Colony formation assay was used to find cell anchorage-independent growth. Cells were mixed with atorvastatin, R115777 or solvent (water) in 1.5 ml of culture medium and seeded into culture dishes (each dose of agent in triplicate). The colony formation was scored after 14 days at 37°C. Percentage of inhibition of colony formation was calculated as compared to the control.

Cell fractionation and Western Blot analysis

Pancreatic tissues or cultured cells treated with atorvastatin and R115777 were lysed in a complete lysis buffers (Santa Cruz Biotechnology, Inc. CA), containing a cocktail of phosphatase, protease, and proteasome inhibitors followed by centrifugation at 900×g at 4°C for 20 min. The supernatants were further centrifuged at 105,000×g at 4°C for 90 min. The cytosolic fractions (supernatant) were collected and kept at -80°C for further analysis. The pellets were washed and the membrane fractions were recovered by centrifugation at 105,000×g at 4°C for 90 min. The membrane pellets were resuspended in 0.25 M sucrose solution with a cocktail of inhibitors and stored at -80°C. The protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce).

Heat-denatured membranous and cytosolic fractions underwent SDS-polyacrylamide gel (4–12% gradient) electrophoresis. The resolved proteins were transferred onto a polyvinylidene fluoride membrane and probed with the primary antibody in blocking buffer at 4°C overnight. After washing with tris-buffer three times, the membrane was incubated with the secondary antibody, and visualized by enhanced chemiluminescence (ECL, Amersham Kit, Arlington Heights, IL). Specific protein band intensity was quantified using Image-Pro Plus.

Illumina Microarray analysis for global gene expression

Microarray analysis was conducted with an Illumina Mouse Ref-8 v1.1 Expression Bead Chip (Illumina, San Diego, CA). Total RNAs were extracted from cultured mouse pancreatic carcinoma cells following treatment with atorvastatin for 48 hours using RNeasy Plus Mini kit (Qiagen, Valencia, CA). RNA quality was checked using an Agilent Bioanalyzer (Santa Clara, CA). An Ambion labeling kit was used for labeling cDNA followed by hybridization to Illumina chips. Scan data was extracted with Illumina Bead Studio and subsequently analyzed using Bioconductor Lumi package²⁹. The data were first variance stabilization transformed and then normalized by a quantile normalization method³⁰. To reduce false positives, probes with measurement values below the background level (detection *P* value <0.01) in all hybridizations were filtered out; 11,168 genes with present count (the number of samples in which the gene was detectable) larger than 0 were kept for subsequent statistical analysis. Genes with significant differential expression levels were identified using analysis of variance with Bayes adjustment of the

variance implemented in Bioconductor Lumi package³¹. To control the effects of multiple testing and reduce false positives, the gene selection of differentially expressed genes was based on a 2.0-fold-change (up or down). The identified genes were then used for the outlined studies.

Ingenuity Pathway Analysis

The sorted lists of up and down-regulated genes that showed expression levels (fold change equal to or more than 2 up or down) containing the Illumina probe set identifiers and their averaged signal log ratios (log2) were loaded into the Ingenuity Pathway Analysis software (Ingenuity System, www.ingenuity.com) to help organize them into known biological pathways, which were called “canonical pathways”. When probe sets redundantly symbolized a single gene, the probe set with the maximum signal log ratio was used in the analysis. Probability scores for each network or functional grouping were calculated as to the chances of the mRNA abundance changes predicting these interaction and networks^{32, 33}.

Quantitative Reverse-Transcribed Real Time-PCR (RT-PCR) analyses

One microgram total RNA was reverse-transcribed into the first strand cDNA using SuperScript III Platinum Two-step qPCR kit with SYBR Green (Invitrogen, CA). The SuperScript III reverse transcriptase is a modified MMLV-derived reverse transcriptase and the SuperScript III reaction mix contains oligo(dT)₂₀, random hexamers, MgCl₂, and dNTPs. The first strand of cDNA was synthesized according to the manufacturer's instructions and the generated cDNA was stored at -20°C until used. Quantitative real-time PCR using SYBR Green assay methods was used to verify the differential expression of selected genes from the results of the array and pathways analysis. The primers for the real-time PCR were designed with Primer Quest software (IDT, Coralville, IA) based on the mouse genomic sequence. The PCR reactions were performed using the MiniOpticon real-time PCR Detection System (Bio-Rad, Hercules, CA). Thermal cycling conditions were 50°C for 2 minutes, 90°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 58°C for 30 seconds. All reactions were run in triplicate with mouse GAPDH gene as reference. The relative mRNA expression levels were calculated by normalizing the testing-crossing threshold (Ct) with each set of samples using the difference in threshold cycles (Ct) between target genes and internal control GAPDH gene, $2^{-\Delta\Delta C_t}$. Primers for real-time PCR are listed in supplement Table 1.

Statistical Analysis

The data was expressed as the mean $\pm$ SE. Statistical significance was tested using SigmaStat Version 1.01 software (Jandel Scientific, San Rafael, CA). Kaplan-Meier Survival Analysis with Log-Rank Test was performed. Tumor incidence was analyzed using the Chi-square test. Normally distributed data was analyzed using Student's t-test, and non-parametric data, such as tumor multiplicity, was analyzed using the Mann-Whitney Rank Sum test.

Results

Increased animal survival in Pan^{kras/p53} mice treated with atorvastatin

Survival is the most significant determinant for evaluating the efficacy of cancer chemoprevention, particularly for the most lethal malignant pancreatic carcinoma. The survival after development of pancreatic adenocarcinoma was determined in Pan^{kras/p53} mice treated with 100 ppm atorvastatin. Kaplan-Meier survival analysis with Log-Rank test was performed, and showed that when the mortality reached to 75%, the average survival in

Pan^{kras/p53} mice fed with the AIN 93M diet alone was 144.9 ± 8.4 days (Mean $\pm$ SE, 95% lower 128.4 & 95% upper 161.4). At this time point, the average survival in *Pan^{kras/p53}* mice fed with the AIN 93M diets supplemented with 100 ppm atorvastatin was 171.9 ± 6.2 days (Mean $\pm$ SE, 95% lower 159.7 & 95% upper 184.1), which was statistically as significant compared to *Pan^{kras/p53}* mice fed the AIN 93M diet alone ($p < 0.05$), as seen Fig. 1A. Therefore, the entire animal experiment was terminated at this time point.

Body weight, and consumption of food and water were monitored and there was no significant difference in mice fed either the AIN 93M diet or the diet containing 100 ppm atorvastatin.

Inhibition of pancreatic carcinogenesis in *Pan^{kras/p53}* mice treated with atorvastatin

Another key marker for evaluating the effect of atorvastatin on pancreatic carcinogenesis was tumor size and volume. As shown in Fig. 1B, the tumor volume/size in mice treated with 100 ppm atorvastatin were significantly decreased in comparison to the control *Pan^{kras/p53}* mice (1093 ± 1386 mm³ versus 1969 ± 1261 mm³, $p < 0.001$).

The pancreatic tumor and its metastases to other organs, as well as the primary tumors in organs other than the pancreas were extensively analyzed histopathologically. Morphologically, pancreatic tumors were identified as pancreatic ductal adenocarcinoma (PDAC) either well, moderate or poorly-differentiated adenocarcinoma (Fig. 2A – 2C), some of which showed spindle cell sarcomatoid features (Fig. 2D). These carcinomas often invade into the adjacent organs such as the duodenum and commonly metastasize to the adjacent lymph nodes, liver (Fig. 2E – 2F) or and spleen (Fig. 2G – 2H). In addition, hematopoietic malignancies such as high grade lymphoma in the spleen (Fig. 2I – 2J) and squamous papilloma of the vagina were also commonly observed. As shown in Table 1, in *Pan^{kras/p53}* mice, 100% (20/20) of animals developed malignant neoplasms including 90% PDAC and 10% neoplasm in organs other than the pancreas. The tumors occurring in extra-pancreatic sites included lymphomas in the spleen, sarcomatoid malignant neoplasms in soft tissue, and squamous cell papilloma in vagina. The detailed morphologic analysis of tumor development in the pancreas as well in the other organs in mice treated with atorvastatin was summarized in Table 1.

To evaluate the chemopreventive effect of atorvastatin on the development of pancreatic carcinoma, reduction of tumor incidence was observed in *Pan^{kras/p53}* mice treated with 100 ppm atorvastatin compared to mice without atorvastatin treatment 75% (15/20) versus 100% (20/20), but the difference did not reached statistical significance ($p > 0.05$).

Inhibition of Ki-67 labeled cell proliferation in pancreatic adenocarcinoma in *Pan^{kras/p53}* mice treated with atorvastatin

Ki-67 labeled cell proliferation was determined immunohistochemically. Compared to the non-atorvastatin-treated *Pan^{kras/p53}* mice (Fig. 2K), marked decrease of Ki-67-labeled cell proliferation in pancreatic carcinoma was observed in mice treated with 100 ppm atorvastatin (Fig. 2L). The proliferation index (% of Ki-67 positive cells in the total tumor cells counted) was further measured and showed $29.5\% \pm 3.5\%$ in *Pan^{kras/p53}* mice and $11.3\% \pm 2.2\%$ in mice treated with 100 ppm atorvastatin ($p < 0.01$).

Inhibition of membrane bound Kras protein and phosphorylated c-raf in pancreatic adenocarcinoma in *Pan^{kras/p53}* mice treated with atorvastatin

Prenylation is a key process for Kras activity by anchoring Kras protein to cell membrane; in other word, determination of membrane bound Kras protein is a crucial biomarker of prenylated Kras protein. Membrane fractions of pancreatic tumor tissues were analyzed for

level of membrane-bound prenylated Kras protein using a Western blot approach, and a membranous protein Flotillin-2 was used as an internal control. A marked decreased level of membrane-bound Kras protein was observed in pancreatic tumor in mice treated with 100ppm atorvastatin compared to the control mice (Fig. 3A). Using β -actin as an internal protein control, a phosphorylated c-raf protein, a gene immediately downstream of Kras, was markedly decreased (Fig.3A). A densitometer analysis demonstrated that with 100 ppm atorvastatin treatment, membrane-bound Kras protein and its downstream phosphorylated-c-raf protein were significantly reduced in mice treated with atorvastatin (Histogram in Fig. 3B, $p<0.05$).

Inhibition of pancreatic cancer cell growth and protein prenylation by atorvastatin and farnesyltransferase inhibitor R115777 in the cultured murine pancreatic carcinoma cells

In vitro cultured mouse pancreatic carcinoma cell lines derived from either primary or metastatic carcinomas from *Par^{Kras/p53}* mice were well established. Two cell lines (P01 and P02) were from primary pancreatic adenocarcinomas obtained from different mice. A metastatic adenocarcinoma cell line (M01; metastatic to liver) was established as well, which was obtained from the same mouse as the P02 cell line. All three cell lines have been cultured *in vitro* for more than two years and with more than 50 passages (the detailed features of cell lines were analyzed and confirmed its neoplastic feature and epithelial origin with active mutant *Kras^{G12D}* and *p53^{R172}* which will be reported in a separate manuscript). A dose-dependent inhibitory effect on cell growth by atorvastatin and farnesyltransferase inhibitor R115777 was observed in all of three pancreatic carcinoma cell lines (M02, P02 and P01) with IC₅₀ doses of 6.8, 8.6 and 12 μ M for M02, P02 and P01 cells by atorvastatin, respectively, and IC₅₀ doses of 12 μ M for P02 cells by R115777, using colony formation assay.

Membrane bound prenylated Kras protein was further analyzed in these cell lines treated with atorvastatin for 48 hours using a Western blot approach and Flotillin-2 as an internal control membrane protein. Membrane bound prenylated Kras protein was significantly reduced by atorvastatin treatment in the dose-dependent manner in P02 murine pancreatic carcinoma cells (Fig.3C). Furthermore, with the β -actin as an internal control protein, Kras downstream signals were also evaluated and showed that the phosphorylation of c-Raf, C-Jun, and p90RSK were significantly decreased in P02 cells treated with atorvastatin in a dose-dependent manner, as shown in Fig. 3C. Similar to Atorvastatin treatment, P02 murine pancreatic carcinoma cells treated with R115777 agent showed a significant reduction of membrane bound prenylated Kras protein in dose-dependent manner, as well a significant reduction of the phosphorylated c-Raf and ERK1/2 proteins (Kras downstream signals), as seen in Fig.3D.

Prenylation was initially believed to be important only for membrane attachment, but the 15-carbon farnesyl moiety alone does not stably anchor proteins to membranes³⁴. Another role for prenylation appears to be its importance in protein-protein interactions³⁵; and the human DNA-J homolog, HDJ2, and nuclear prelamin A are such proteins and are commonly used as the biomarker for the evaluation of protein prenylation. As shown in Fig. 3C, non-farnesylated HDJ-2 and nuclear non-farnesylated prelamin A proteins were markedly increased following an atorvastatin treatment for 48 hours. Furthermore, trimethylated histone 3, a nuclear lamin/prelamin A downstream signal was markedly inhibited after atorvastatin treatment.

Modulation of global gene expression profile in pancreatic carcinoma cells treated by atorvastatin using Illumina microarray method

Modulation of global gene expression profiles was analyzed using P02 murine pancreatic carcinoma cells treated with atorvastatin and the Illumina microarray approach. A total of 132 genes were either up or down-regulated by atorvastatin treatment based on the cut-off of >2-fold change from the original 11,168 genes filtered and kept for subsequent statistical analysis. Of the 132 differentially expressed genes, 16 were up-regulated and 1 was down-regulated in P02 cells treated with 3 μ M atorvastatin, while 96 genes were up-regulated and 19 down-regulated in P02 cells treated with 10 μ M atorvastatin, as seen in Fig.4A1 and 4A2 (the details of these genes were summarized in the supplementary data of Table 1 and 2). Ingenuity pathway analysis of the 132 genes showed that these genes were distributed according to function and disease and were, in particular, associated with cancer, lipid metabolism, cellular growth/proliferation, cell cycle, as well as chromatin assembly/disassembly, angiogenesis, inflammation and methylation with statistical significance ($p < 0.0001$).

There are several studies indicating that atorvastatin is a strong anti-inflammatory and anti-carcinogenic agent that is independent of inhibiting HMG-CoA reductase. The genes of *dnmt1*, *vcam1*, *c-fos*, *p21waf* and *Cyp51A1* as well as soluble epoxide hydrolase (sEH) regulated by atorvastatin were of particular interest. *Cyp51A1*, sEH, and vascular adhesion molecule 1 (*vcam1*) are involved in the process of inflammation³⁶. With quantitative Real-Time PCR assay (details on primers was shown in supplementary Table 3), *dnmt1*, *vcam1* and *c-fos* as well as sEH were confirmed to be significantly down-regulated while *p21waf* and *cyp51A1* to be up-regulated (Fig. 4B). The up-regulation of *cyp51A1* and *waf1p21*, and the down-regulation of sEH were further demonstrated in P02 mouse pancreatic carcinoma cells treated with 3 and 10 μ M atorvastatin using a western blot assay (Fig.4C).

Since *Cyp51A1*, sEH, and vascular adhesion molecule 1 (*vcam1*) are involved in the process of inflammation, especially for inflammatory cell infiltration³⁶, myeloperoxidase (MPO)-labeled neutrophil infiltration in the areas of chronic pancreatitis was analyzed immunohistochemically. As compared to Pan^{*Kras/p53*} control mice (Fig.4D1), a marked reduction of MPO-labeled neutrophil infiltration was observed in mice treated with 100 ppm atorvastatin (Fig.4D2) and further semi-quantitative analysis showed a significant decrease of MPO-labeled neutrophils in chronic pancreatitis (59.8 ± 10.3 vs. 21.1 ± 18.6 neutrophils/HPF, $n=5$ mice/group, $p=0.008$).

Discussion

Pdx1-Cre recombination for the activation of a knock-in mutant allele of *Kras* and *p53* genes is a robust mouse model mimicking the multiple genetic alterations seen in human pancreatic carcinogenesis. Pan^{*Kras/p53*} mice have concomitant endogenous expression of mutant *Kras*^{G12D} and *p53*^{R172} in the pancreas with 100% of mice developing malignant neoplasms including pancreatic ductal adenocarcinomas. Due to the highly lethal pancreatic malignancy and aggressive behavior, the lifespan or survival of Pan^{*Kras/p53*} mice from pancreatic carcinogenesis would be a significant endpoint determinant or marker for evaluating the chemopreventive effects of various agents against pancreatic carcinogenesis. To our knowledge, we are the first group to demonstrate a significant increase in animal survival following pancreatic carcinoma development in Pan^{*Kras/p53*} mice treated with 100 ppm atorvastatin.

It is crucial to design a dosage of chemopreventive agents used in animal models comparable to the dosage in humans. In general, the animal dose should not be extrapolated to a human equivalent dose (HED) by a simple conversion based on body weight due to

significant differences in multiple aspects including metabolic rates²⁶. For the more appropriate conversion of drug doses from animal studies to human studies, the body surface area (BSA) normalization method and nutrient density are commonly used to compare dosage differences between rodents and humans^{25, 26}. BSA correlates well across several mammalian species with several parameters of biology, including oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function. The FDA's correct extrapolation of animal dose to human dose is stated as calculated by this formula: "To convert the dose used in a mouse to a dose based on surface area for humans, multiply the animal dose by the Km factor for a mouse and then divide by the Km factor for a human²⁶." The animal Km is 3 in mice and the human Km is 37. Therefore, in calculating the human equivalent dose (HED) based on the formula: $\text{HED (mg/kg)} = \text{animal dose (mg/kg)} \times (\text{Animal Km}/\text{Human Km}) = \text{animal dose (mg/kg)} \times (3/37) = \text{animal dose (mg/kg)} \times 0.081$. Based on this formula, the step by step calculation of appropriate atorvastatin dose in mouse is as follows: 1) the FDA recommendations for atorvastatin dosing allow for up to 80mg per day in patients and states that dosing should start at 40mg/day in patients with LDL levels over 45%. In a hypothetical 75kg patient, a dosing of 80mg/day works out to be a human dose of 1.06mg/kg of atorvastatin. Substituting that human dose of 80mg/day (1.06mg/kg) into the equation is $1.06 \text{ mg/kg} = \text{animal dose (mg/kg)} \times 0.081$; therefore, the animal dose of atorvastatin based on the maximum human dose of 80mg would be 13.08 mg/kg. 2) The average body weight of a mouse is about 25 grams. To solve for the dosage in a mouse based on that body weight, the calculation would simply be: $(13.08 \text{ mg/kg}) \times \text{weight of mouse in kg} = \text{dose in milligrams}$, that was $(13.08 \text{ mg/kg}) \times (0.025 \text{ kg}) = 0.328 \text{ mg}$. Thus, the mouse can safely receive up to 0.328 mg of atorvastatin per day. 3) Translating this dose into the actual diet concentration - the average mouse consumes 2 grams (0.002 kg) of food per day, and the concentration of the atorvastatin in the diet should be $0.328 \text{ mg atorvastatin}/0.002 \text{ kg food} = 164 \text{ mg atorvastatin/kg food}$, that is equal to 164 ppm of atorvastatin in the mouse diet. Finally, our mice were receiving an atorvastatin dose of 100 ppm that was well below the 164ppm "maximum" dose of atorvastatin based on human clinical recommendations and was the most close to a comparable dosage to a clinically acceptable intermediate dose range (40 mg per day of atorvastatin) On the other hand, BSA method also well correlates nutrient density calculation²⁵. A human dosage of 10–40 mg per day of atorvastatin for a 75kg human consuming a 2000 kcal/kg diet represents a nutrient density of 5 or 20 μg of atorvastatin per Kcal. For a 25g mouse consuming 2g of AIN93M diet (3.6 Kcal/g), 100 ppm of atorvastatin in the diet is a nutrient density of 27.8 μg per Kcal. Since mice have approximately a 10 times higher basal metabolic rate compared to humans, a nutrient density of 27.8 μg per Kcal of atorvastatin in mice likely corresponds to a low dose or a human dosage of 10 mg per day³⁷. Thus, the dose used in the animals was not the excessive maximum dose in human and should be valid in comparing the efficacy of the atorvastatin in the animals to humans^{38, 39}.

Increase of survival from pancreatic cancer development is the goal of cancer chemoprevention but obviously it would be a most challenge and difficult endpoint marker due to long-term expensive experiment or almost impossible trial. The commonly used surrogate endpoint biomarkers are tumor incidence, multiplicity and volume/size in animal experiment/s. In the present studies, in addition to the increase of survival, a significant reduction in the tumor size/volume and inhibition of Ki-67-labeled cell proliferation were observed in tumors in mice treated with atorvastatin. The plausible mechanisms for the inhibition of tumor growth by atorvastatin would include: 1) suppression of the transgenic mutant Kras or p53 activity, 2) inhibition of the process of protein prenylation via targeting the mevalonate metabolic pathway and HMG-CoA reductase, or 3) affecting other biologic/metabolic process independent of HMG-CoA reductase inhibition, such as the inhibition of inflammation.

Using specific PCR and p53 immunohistochemistry approaches, the activation of knock-in mutant *Kras* and *p53* genes by Pdx1-cre recombination was identified in the pancreatic tumors and there was no difference in these gene activities in the mice fed either AIN93M diet or diet containing 100ppm atorvastatin (in supplementary data, Fig. 1-suppl).

Targeting the HMG-CoA reductase and mevalonate metabolic pathway by atorvastatin is the crucial mechanism of inhibiting carcinogenesis. The isoprenoid farnesyl pyrophosphate (FPP) and geranyl-geranyl pyrophosphate, as products of the mevalonate (MVA) metabolic pathway, are involved in the post-translational lipid modification of proteins, called protein prenylation. Using freshly collected pancreatic tumor tissues and *in vitro* cultured pancreatic carcinoma cells as well as a western blot approach, prenylation in several key proteins including *Kras*, nuclear lamin A and human DNA-J homolog, HDJ2 (typical biomarkers of prenylated proteins) was inhibited by atorvastatin treatment. Furthermore, inhibition of *Kras* activated down-stream signals, including phosphorylated c-Raf, phosphorylated C-Jun, and phosphorylated p90RSK, were observed. A parallel experiment in the *in vitro* cultured pancreatic carcinoma cells treated with farnesyltransferase inhibitor R115777 showed similar pattern and effect on the reduction of membrane-bound prenylated *Kras* protein and its down-stream signals. Since *Kras* is one of the most important oncogenes involved in early pancreatic carcinogenesis and is a central gene initiating carcinogenesis in the *Pan^{Kras/p53}* mouse model, our results demonstrate that inhibition of *Kras* protein prenylation and its activity by atorvastatin is an important mechanism for inhibiting pancreatic carcinogenesis.

Nuclear lamin and human DNA-J homolog HDJ2 are not only two typical biomarkers for evaluating prenylation, but also play a crucial role in cell biology. Nuclear lamins support the nuclear envelope and provide anchorage sites for chromatin; they are also involved in DNA synthesis, transcription, and apoptosis. The prenylation/farnesylation of prelamin A is important for its targeting of the nuclear envelope. The complete processing of farnesyl–prelamin A to mature lamin A is critically important. An accumulation of farnesyl–prelamin A (as occurs with a deficiency in the prelamin A processing enzyme Zmpste24) causes dramatically misshapen nuclei and a Progeria-like disease phenotype or neoplastic appearance of nuclear atypia⁴⁰. Obviously, inhibition of farnesyl–prelamin A would significantly reduce the frequency of nuclear atypia and improve aberrant chromatin remodeling. There is a suggestion that prelaminA might be prenylated efficiently by both geranylgeranyltransferase and farnesyltransferase. Thus, HMG-Co A reductase, an upstream key enzyme of both geranylgeranyltransferase and farnesyltransferase would be more efficient to inhibit lamin A prenylation. Our results showed the lamin A prenylation as well as its down-stream signal (trimethylated histone-3) were markedly decreased in pancreatic carcinoma cells treated with atorvastatin. All of these results indicate that significant inhibition of protein prenylation by atorvastatin may play a crucial role in inhibiting pancreatic carcinogenesis.

The human DNA-J homolog, HDJ2, is a co-chaperone containing a cysteine-rich zinc finger domain and a mitochondrial protein. HDJ2 is also a heat-induced and farnesylated protein that is apparently involved in mitochondrial protein import in mammals⁴¹. It has been reported that HDJ2 is involved in various cellular processes including intracellular protein trafficking and folding, particularly in cooperation with heat shock protein 70 (Hsp70), as well as reduction/oxidation reactions and cell apoptosis. Thus, HDJ2 as a heat shock protein is important in the process of stress, inflammation and carcinogenesis, as well as for the increased radiation therapeutic effect for cancer treatment. Here, the functional significance for the inhibition of HDJ2 protein prenylation needs to be investigated further.

To further look for a mechanism of inhibiting pancreatic carcinogenesis independent of HMG-CoA reductase inhibition, global transcriptional gene array analysis was performed and showed 132 genes were modulated when a 2-fold change was employed for pancreatic carcinoma cells treated with atorvastatin. In addition to the expected genes involved in cellular metabolic process/lipid metabolism and cardiovascular disease, the genes involved in cell cycle regulation, chromatin assembly/disassembly, angiogenesis, inflammation and methylation were observed. Modulation of several selected key gene expression by atorvastatin was further confirmed with quantitative RT-PCR and Western blot analysis, including cell cycle control genes (Waf1p21), chromatin assembly and disassembly genes (lamin A and EZH2), transcriptional factor (C-fos), inflammation and angiogenesis genes (cyp51A1, sEH, VEGF and VCAM1) and DNA methylation (DNMT1). These findings provide a clue and opportunity to further explore in-depth mechanism of anti-tumorigenesis by atorvastatin in the future.

Inhibition of inflammation is one of the many pleiotropic effects of statins that are independent from HMG-CoA reductase inhibitor, and there is increasing evidence for the potential use of statins in preventing and treating inflammation. The mechanism by which statins modulate the immune response is complex, and possible mechanisms include that statins significantly reduce leucocyte adhesion to the endothelium by down-regulation of surface expression of endothelial cell adhesion molecule P-selectin and VCAM-1, CD11b, and CD18 and by inhibition of lymphocyte function antigen-1 (LFA-1)-mediated leucocyte adhesion⁴² and decrease macrophage-mediated chronic inflammation⁴³. Our results showed that inhibition of inflammatory cell infiltration in chronic pancreatitis by atorvastatin appeared consistent with the modulation of several key inflammation-related genes, in particularly to down-regulate soluble epoxide hydrolase (sEH) and up-regulate P450 51A1. P450 51A1 and sEH are the key enzymes involved in p450-mediated arachidonic acid metabolic pathway, especially for the production and hydrolation of the anti-inflammatory epoxyeicosatrienoic acids (EETs)^{36, 44}. Anti-inflammatory activity by EETs is mainly via preventing leukocyte adhesion to the vascular wall and infiltrating into inflamed tissues through the reduction of cytokine-induced VCAM-1 expression and inhibition of transcription factor NF-kB and IKK kinase⁴⁵. sEH hydrolyzes EETs to dihydroxyeicosatrienoic acid (DHETs) that deactivate EET biologic anti-inflammatory functions⁴⁴. Thus, upregulation of P450 51A1 and downregulation of sEH by statin to stabilize and increase EETs would be a crucial mechanism of anti-inflammation and anti-carcinogenesis.

In conclusion, treatment with atorvastatin in the *Pan^{kras/p53}* mice resulted in significantly increased animal survival following pancreatic carcinoma development and a significant inhibition of pancreatic carcinoma growth. Mechanistically inhibition of protein prenylation in several key proteins including Kras, nuclear lamin A and HDJ-2 is a crucial event in inhibiting pancreatic carcinogenesis by atorvastatin. Transcriptional gene array analysis revealed 132 genes regulated by atorvastatin in pancreatic carcinoma. Targeting P450 51A1, sEH and VCAM1 by atorvastatin is a new mechanism of anti-inflammation and anti-carcinogenesis. These findings indicate that atorvastatin is a potential chemotherapeutic agent in the prevention of human pancreatic carcinoma development in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Animal survival and tumor volume/size in *Pan^{kras/p53}* mice fed the diet with or without 100 ppm atorvastatin. A) Kaplan-Meier Survival Analysis with Log-Rank Test for animal survival. B) Distribution of tumor volume.

Fig.2.

Representative morphologic features of primary and metastatic malignant neoplasms in the pancreas and other organs in *Pan^{kras/p53}* mice: A) low power magnification of pancreatic carcinoma in the highlighted areas surrounded by duodenum and adjacent non-neoplastic pancreas; B) a well-differentiated pancreatic adenocarcinoma with glandular/tubular structure; C) moderately differentiated pancreatic adenocarcinoma with cribriform pattern; and D) poorly differentiated and sarcomatoid carcinoma of the pancreas with highly atypical nuclei; E) metastatic pancreatic carcinoma in the liver; F) high power magnification of metastatic carcinoma of square area of photo E. G) metastatic pancreatic carcinoma in the spleen; H) high power magnification of metastatic carcinoma of square area of photo G. I) low power magnification of spleen showing diffuse involvement by lymphoma; J) high power magnification showing diffuse large cell lymphoma with highly atypical nuclei and multiple mitoses. K) Ki-67 labeled cell proliferation in pancreatic carcinoma in *Pan^{kras/p53}* mice using an immunohistochemical approach, and L) Ki-67 labeled cell proliferation in pancreatic carcinoma in *Pan^{kras/p53}* mice treated with 100 ppm atorvastatin.

Fig.3.

Determination of prenylated proteins and their down-stream signals in the pancreatic tumors in *Pan^{kras/p53}* mice fed AIN 93M diet with or without 100 ppm atorvastatin and in P02 murine pancreatic carcinoma cells treated with atorvastatin or R115777 using western blot approach. **A)** Analysis of membrane-bound Kras protein and phosphorylated c-raf protein in *Pan^{kras/p53}* mice (Lane 1–2: pancreatic carcinoma of *Pan^{kras/p53}* control mice; Lane 3–4: pancreatic carcinoma of *Pan^{kras/p53}* mice treated with 100 ppm atorvastatin). **B).** Densitometric analysis of membrane-bound Kras and phosphorylated c-raf proteins: a significant decrease of specific protein band intensities including membrane-bound Kras protein and phosphorylated c-raf protein in pancreatic tumors treated with atorvastatin ($p < 0.05$). **C)** Analysis of membrane-bound Kras protein and its downstream signals as well other prenylated proteins in P02 murine pancreatic carcinoma cells treated with atorvastatin in the dose range of 0, 10, 20 and 40 μM for 48 hours: atorvastatin-treated cells showed a dose-dependent reduction of membrane-bound Kras protein and phosphorylated c-raf, c-jun, and P90RSK, as well an increase of non-prenylated protein band in HDJ2 and nuclear lamin A proteins and a marked decrease of trimethylated histone-3. **D)** Analysis of membrane-bound Kras protein and its downstream signals in P02 murine pancreatic carcinoma cells treated with R115777 in the dose range of 0, 5, 10 and 20 μM for 48 hours: R115777-treated cells showed a dose-dependent reduction of membrane-bound Kras protein and phosphorylated c-raf, Erk1/2 and c-jun. Flotillin-2 and β-actin were used as internal house-keeping protein controls

Fig.4.

Global gene expression profile in murine pancreatic carcinoma cell P02 treated with atorvastatin and immunohistochemical analysis of myeloperoxidase-labeled inflammatory cells (neutrophils) in chronic pancreatitis in *Pan^{kras/p53}* mice treated with atorvastatin. **A)** MA plot of a global view of gene up and down-regulated in mouse P02 pancreatic carcinoma tumor cells treated with 3 μ M (A1) and 10 μ M (A2) of atorvastatin, compared with cells without treatment: MA plot indicates the relationship between the observed fold changes and the expression mean of cells treated with atorvastatin, comparing with cells without non-treatment. Each gene is represented as a dot. The red dots represent the gene with fold-change larger 2 folds (number below 0 is down-regulated fold change, higher than 0 is up-regulated fold changes at the y-axis). **B)** Quantitative real-time PCR analysis for the selected genes in response to 3 μ M (white bar) and 10 μ M (black bar) atorvastatin treatments, the transcriptional changes were expressed relative to the housekeeping gene, GAPDH. The mean value $\pm$ SD of three independent experiments was shown. **C)** Western blot analysis: further confirmation of the changes of Waf1p21, Cyp51a1 and sEH protein levels in mouse pancreatic carcinoma cells after treatment with 3 μ M and 10 μ M of atorvastatin for 48 hrs. β -actin was used as an internal loading control. **D)** Immunohistochemistry of myeloperoxidase-labeled inflammatory cells (neutrophils) in chronic pancreatitis adjacent to pancreatic carcinoma of *Pan^{kras/p53}* mice fed either control diet (D1) or the diet with 100 ppm atorvastatin (D2).

Table 1

Morphologic analysis of tumor development in the pancreas as well in the other organs

Organ	Lesions	Control Diet	100 ppm Atorvastatin
Pancreas	Adenocarcinoma	90.0% (18/20)	75% (15/20)
	Adenocarcinoma with sarcomatoid feature	11.1% (2/18)	20.0% (3/15)
Adenocarcinoma directly invasion into adjacent organs	Duodenal	16.7% (3/18)	6.7% (1/15)
	Kidney	0.0% (0/18)	6.7% (1/15)
Metastasis to other organs	Liver	11.1% (2/18)	13.5% (2/15)
	Lymph Node	5.6% (1/18)	6.7% (1/15)
	Spleen	5.6 (1/18)	0%(0/15)
Tumors occurred in other Organs	Lymphoma in spleen	15.0% (3/20)	5.0% (1/20)
	Vaginal Squamous Papiloma	20.0% (4/20)	10.0% (2/20)
	Sarcoma in subcutaneous tissue	5.0% (1/20)	0.0% (0/20)
	Adenoma in Colon	10.0% (2/20)	5.0% (1/20)
Overall Tumor Incidence		100.0% (20/20)	75% (15/20)