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TITLE: Targeting the Mevalonate Pathway to Reduce Mortality from Ovarian Cancer

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| 13. SUPPLEMENTARY NOTES | | | | | | |
| 14. ABSTRACT The primary purpose was to evaluate whether statins, a well-known cholesterol-lowering agent, could improve survival in women with epithelial ovarian cancer. Aim 1: a) prospectively examine whether statin use reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer after adjustment for stage, grade, treatment, histologic subtype, co-medication use, type of surgery and type of hospital. Statin use will be compared to non-users as well as users of other lipid lowering agents; and b) test whether the association is modified by: i) dose and duration, ii) timing of the intervention (pre-diagnosis versus post diagnosis use), iii) histologic subtype and iv) degree of adherence. Aim 2:a) assess the anti-tumor effect of lovastatin, (a commonly prescribed statin) alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3, and A2780 cells and; b) determine the molecular mechanism by which lovastatin inhibits tumor growth. The clinical and translational impact of this project are substantial because unlike other potential new drug treatments, statins are already in wide use, have been shown to have very low toxicity and could therefore be put quickly into practice (if clinical trials confirm their efficacy) and at low cost. | | | | | | |
| 15. SUBJECT TERMS: cancer mortality, cholesterol-lowering drugs, disease progression, epithelial ovarian cancer, lovastatin, Mevalonate Pathway, prescription drugs, statins, survival, transgenic mouse tumor model | | | | | | |
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Section I – INTRODUCTION:

The overall project goal was to evaluate whether the addition of statins, well-known cholesterol-lowering agents, would improve survival in women with epithelial ovarian cancer.

Our hypothesis was that both pre-diagnostic and post-diagnostic statin use would improve survival among women with epithelial ovarian cancer and that a higher dose or longer duration of use will be associated with a greater reduction in overall mortality. We also hypothesized that the anti-tumor effects of statin are mediated by a pathway that included enzymes that are involved in farnesylation.

To test our hypothesis, we undertook a multidisciplinary approach that included conducting both a large epidemiological study and preclinical studies.

In Aim 1 we tested our hypothesis in a national dataset from Finland of 10062 cases of epithelial ovarian cancer to look at statin use to explore their impact on reducing cancer specific and overall mortality and if other factors (i.e. dose and duration, timing of the intervention (pre versus post diagnosis use), histologic subtype, and patterns of adherence) modify the association. Initially we used conventional survival methods to analyze the data as proposed in our project but we then became aware of some novel sophisticated methods that could be used to minimize bias due to time varying confounding which can be inherent in observational studies. This meant we had to develop new programs etc. and re-run all the analysis. We are almost done with that and in process of writing up the manuscript and putting the figures together. This was a major task but worthwhile doing as it really altered some of the estimates and therefore is a significant problem that is not accounted for in the published literature. We are only aware of one recent study examining statin use and breast cancer that has used this approach. Using standard cox proportional hazard and cumulative incidence models incorporating time varying confounders we observed that patients who initiated statin use after diagnosis of ovarian cancer was associated with a 70% reduction in mortality (HR = 0.29, 95%CI 0.25, 0.34) but based on the new approach using marginal structural models to model time varying changes in medical conditions associated with the initiation of statin use we obtained what would be a more realistic estimate a 35% reduction in mortality (weighted HR= 0.65, 95% CI 0.57-0.74). Although less of an issue when we used the standard approach to examine the association between ovarian cancer patients who initiated statin use prior to their diagnosis of ovarian cancer we observed a 23% reduction in mortality (HR=0.77, 95%CI 0.71, 0.84) but when we used the new methods the weighted HR = 0.63, 95%CI 0.58, 0.70). We are now in process of updating all the many analyses using time varying marginal structural models for the paper. This will strengthen the paper significantly by producing results closer to that of a clinical trial. We expect the updated analysis to be completed in the next few weeks now that we have all the programming done and expect to have the manuscript submitted within the next month. We would be happy to provide a further update at that time.

In Aim 2 we demonstrated that lovastatin significantly reduced the development of STICs in mogp-TAg mice and inhibited ovarian tumor growth in the mouse xenograft model. Knockdown of prenylation enzymes in the mevalonate pathway recapitulated the lovastatin-induced antiproliferative phenotype.

See “**Research Summary**” for Aim 1 and Aim 2 in Section III. B. below for comprehensive overview of the results for the work conducted as outlined on the statement of work.

Section II – KEYWORDS:

Cancer mortality
Carboplatin/paclitaxel
Cholesterol-lowering drugs
Disease progression
Drug exposure
Epithelial Ovarian Cancer
Finnish Cancer Registry
Lovastatin

Mevalonate pathway
 Prescription drugs
 Social Insurance Institute of Finland
 Statins
 Survival
 Transgenic mouse tumor model
 Bisphosphonates

Section III – ACCOMPLISHMENTS:

A. What were the major goals of the project?

| SPECIFIC AIM 1: | Timeline | Status |
|--|---------------------------|-------------------------------|
| 1a) examine whether statin use, reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer; 1b) test whether the association is modified by dose and duration, timing of the intervention (pre vs. post diagnosis use), histologic subtype, and patterns of adherence after adjusting for prespecified covariates. | Start date 9/30/14 | |
| Major Task 1: Obtain approvals from Johns Hopkins SPH IRB, DOD HRPO, MTA and USAMRAA. | Months | Completed Year 1 |
| Subtask 1: Protocol development and submission to Johns Hopkins SPH IRB for review and approval. | 0-2 | Completed Year 1 |
| Subtask 2: Submission of Johns Hopkins IRB approval and related material for DOD's HRPO review and approval. | 0-2 | Completed Year 1 |
| Subtask 3: Develop MTA- Johns Hopkins & Tampere University, Finland. | 0-3 | Completed Year 1 |
| Major Task 2: Request for data variables to be studied. | | Completed Year 1 |
| Subtask 1: Submit proposal that includes a specific data request to Finnish Cancer Registry so they can identify variables. | 0 | |
| 1. Proposal approval and data request completed. | 4-5 | Completed Year 1 |
| Major Task 3: Clean data, generate study specific variables and analyze dataset on statin use and mortality among OV CA cases as well as associations/ modifications factors such as dose, duration, pre/post-diagnosis use, histologic subtype and patterns of adherence. | | Completed Year 3 (NCE) |
| Subtask 1: Data preparation which includes cleaning, extracting medication use from prescription records, recoding data, generating new study specific variables. | 4-9 | Completed Year 3 (NCE) |
| Subtask 2: Conduct primary data analysis regarding stains and mortality among OV CA cases & interpret results (aim 1a). | 9-13 | Completed Year 3 (NCE) |
| Subtask 3: Conduct secondary data analysis regarding associations or modification of statin use & adherence and a number of sensitivity analyses (aim 1b). | 14-20 | Almost completed (NCE) |
| Subtask 4: Prepare and submit an abstract to a national | 14-20 | To be submitted to |

| | | |
|---|--------------------|---|
| professional meeting (either AACR or ASCO). | | ASCO in Jan 2018 |
| Subtask 5: Prepare and submit manuscript for journal submission and begin developing subsequent study based on results. | 16-24 | In preparation |
| 1. Main data analysis completed. | 14-16 | Completed Year 3 (NCE) |
| 2. Secondary analysis completed. | 14-20 | Completed Year 3 (NCE) |
| 3. Presentation of data at national professional meeting (either AACR or ASCO). | 14-20 | To be submitted to ASCO in Jan 2018 |
| 4. Journal manuscript submitted (i.e. Cancer Research or JCO). | 22-24 | In preparation |
| 5. Data used in support of applying for further funding to study new agents in the mevalonate pathway. | 22-24 | Will be used in upcoming for RO1 application and future DOD application |
| SPECIFIC AIM 2: 2a) access anti-tumor effects of lovastatin alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3, OVCAR5 cells; 2b) determine molecular mechanism by which lovastatin inhibits tumor growth. | Start date 9/30/14 | |
| Major Task 1: Obtain approvals from Johns Hopkins Animal Care and Use Committee (IACUC) and the DoD Animal Care and Use Review Office (ACURO). | | Completed Year 1 |
| Subtask 1: Develop and submit proposal to JH IACUC for review. | 0-4 | Completed Year 1 |
| Subtask 2: Submit documents for DoD ACURO for review. | 0-4 | Completed Year 1 |
| 1. Local JH IACUC Approval & DOD ACURO Approval. | 2-4 | Completed Year 1 |
| Major Task 2: In vivo anti-tumor study. | | Completed Year 2 |
| Subtask 1: Establish ovarian cancer cell lines with luciferase-expressing constructs (OVCAR3, SKOV3, & OVCAR5 cells.) Cell line source: ATCC | 0-4 | Completed Year 1 |
| Subtask 2: Purchase athymic <i>nu/nu</i> nude mice (n=120) and set ready for mouse tumor model. Mouse: purchased from Harlan Laboratories | 5,13 | Completed Year 2 |
| Subtask 3: Perform viable surgery by orthotopic injection ovarian cancer cells into mouse ovarian bursa. | 5,13 | N/A |
| Subtask 4: Treat the mice with lovastatin or vehicle control and monitor tumor load using live imaging system. | 6-8, 14-16 | Completed Year 2 |
| Subtask 5: Perform immunohistochemistry to study the expression of proliferation, autophagy, apoptosis markers, as well as other markers identified in our pilot study. | 9-12, 17-20 | Completed Year 2 |
| 1. Determine the anti-tumor effect of lovastatin alone or combination with conventional chemotherapeutic agents. | 5-20 | Completed Year 2 |

| | | |
|---|-------|------------------|
| 2. Identify biomarkers associated with statin treatment in cancer cells. | 5-20 | Completed Year 2 |
| Major Task 3: Conduct gene knockdown and enzyme inhibitor study to determine which sub pathway(s) is mainly responsible for the anti-tumor effects of lovastatin. | | Completed Year 2 |
| Subtask 1: Purchased siRNAs targeting the enzymes belonging to the three sub pathways in the mevalonate metabolism. | 3-4 | Completed Year 1 |
| Subtask 2: Purchase small molecule enzyme inhibitors of the mevalonate sub pathways. | 3-4 | Completed Year 1 |
| Subtask 3: Perform gene knockdown using siRNA in ovarian cancer cell lines (same as described in Task 2). Determine the effects of lovastatin on cellular proliferation, autophagy, and apoptosis as well as the expression of new markers identified in our pilot study. | 4-16 | Completed Year 1 |
| Subtask 4: Determine the effects of inhibitors on the phenotypes described in Subtask 3 in the same panel of ovarian cancer cell lines. Compare the alterations of phenotypes between 3 and subtask 4. | 4-16 | Completed Year 1 |
| 1. Demonstrate the effects of knockdown in those genes regulating mevalonate pathway. | 3-16 | Completed Year 1 |
| 2. Demonstrate the effects of enzyme inhibitors for those proteins regulating the mevalonate pathway. | 3-16 | Completed Year 2 |
| 3. Demonstrate that mevalonate pathway is essential for cellular survival and growth in ovarian cancer cells. | 3-16 | Completed Year 2 |
| Major Task 4: Final phase: data analysis, summary, and publication. | | Completed Year 2 |
| Subtask 1: Analyze and summarize data to support or refute our hypothesis. | 13-24 | Completed Year 2 |
| Subtask 2: Prepare manuscript(s) for journal submission. | 13-24 | Completed Year 2 |
| 1. Presentation of data in meetings or conferences such as AACR annual meeting and others. | 13-24 | Completed Year 2 |
| . Publication of data. | 13-24 | Completed Year 2 |
| 3. Generating research findings that can be used in support of applying for further funding opportunity. | 13-24 | Completed Year 2 |

B. What was accomplished under these goals?

AIM 1:

Aim 1a) examine whether statin use, reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer; 1b) test whether the association is modified by dose and duration, timing of the intervention (pre versus post diagnosis use), histologic subtype, and patterns of adherence after adjusting for pre-specified covariates.

Major Task 1: Obtain approvals from Johns Hopkins SPH IRB, DOD HRPO, MTA and USAMRAA.

Task 1-1: Protocol development and submission to Johns Hopkins SPH IRB for review and approval.

Progress: Completed Year 1

A protocol was submitted and reviewed by the Johns Hopkins SPH IRB and determined that the proposed activity described in the protocol involved secondary data analysis of existing de-

identified/de-linked, not publicly available datasets, and that we were not involved in the original data collection. As such, the proposed activity did not qualify as human subjects research as defined by DHHS regulations 45 CFR 46.102, and did not require IRB oversight.

Task 1-2: Submission of Johns Hopkins IRB approval and related material for DOD's HRPO review and approval.

Progress: Completed Year 1

HRPO reviewed the Johns Hopkins SPH IRB Determination Letter and concurred and sent a "*Research Not Involving Human Subjects Determination Memorandum*" on 5/28/14 stating the project may proceed with no further requirement for review by the HRPO.

Task 1-3: Develop MTA- Johns Hopkins & Tampere University, Finland

Progress: Completed Year 1

A fully executed MTA/Data Use Agreement was put in place 1/5/15. Additionally, we have a signed Commission Decision C (2010) 593 Standard Contractual Clause (processors).

Major Task 2: Request for data variables to be studied.

Task 2-1: Submit proposal that includes a specific data request to Finnish Cancer Registry so they can identify variables.

Progress: Completed Year 1

Approval was granted 12/23/14 from the Finland National Institute for Health & Welfare (THL) to obtain data for ovarian cancer cases registered in the Finnish Cancer Registry (FCR) during 1995-2013. Information on the cancer case's children and 1st degree female relatives, in addition to information on emigrations and possible death was requested from the Finnish Population Register for linkage to FCR to obtain information on the 1st degree relatives' breast and ovarian cancer diagnoses. This was merged with lifetime information on drug purchases and chronic diseases from the Social Insurance Institute (SII) of Finland. Additionally, information on co-morbidity from the hospital discharge (HILMO) was linked to the data.

Major Task 3: Clean data, generate study specific variables and analyze dataset on statin use and mortality among OV CA cases as well as associations/ modifications factors such as dose, duration, pre/post-diagnosis use, histologic subtype and patterns of adherence.

Task 3-1: Data preparation that includes cleaning, extracting medication use from prescription records, recoding data, and generating new study specific variables.

Progress: Completed Year 3 (NCE)

The data from various national organizations was merged together. The data consists of materials combined from the following health care register sources: 1) the Finnish Cancer Registry (information on ovarian cases); 2) the Social Insurance Institution of Finland (information on drug purchases and special reimbursements); 3) Finnish Population Register Center (information on born children and 1st level female siblings, emigration and death); and 4) patient register, HILMO (information on co-morbidity).

We have approximately 3000 more ovarian cancer cases than initially estimated which enabled us to conduct more robust sub-analyses. Our total number of cases was 12,122 compared to 7886, which was originally projected. These women were diagnosed with ovarian cancer between 1/1/1995 - 12/31/2015.

We also have prescription data for those same years on all cases. In our cohort the women appeared to have used 8 different types of statins based on distinct ATC drug codes. The total number of prescriptions for statin drugs is 81,572. Breaking this down further 3389 cases (28%) has filled at least one prescription for statins. We estimated that the prevalence of statin use would be 15% and therefore we should have more than adequate power to complete these analyses.

Task 3-2: Conduct primary data analysis regarding stains and mortality among OV CA cases and interpret the results (aim 1a).

Progress: Completed Year 3 (NCE)

Task 3-3: Conduct secondary data analysis regarding associations or modification of statin use & adherence and a number of sensitivity analyses (aim 1b).

Progress: Completed Year 3 (NCE)

Task 3-4: Prepare and submit an abstract to a national professional meeting (AACR or ASCO).

Progress: To be submitted to ASCO in Jan 2018

Task 3-5: Prepare and submit manuscript for journal submission and begin developing subsequent study based on results.

Progress: In preparation

Aim 1: Below we present the main results of our analyses and illustrate the importance of using time varying marginal structural models instead of the standard survival analysis methods to minimize confounding by indication that is inherent in observational studies with the ultimate goal of approximating the results of a clinical trial. We made this change as we were concerned about the difference in characteristics related to statin use between the groups and was not convinced that adjusting alone which is the standard approach would sufficiently reduce any bias. Previously we had used propensity score matching this new method is a bit more advanced as it takes into consideration changes in time of these factors. To conduct this new analysis required much more data management and programming efforts than initial proposed but we are very happy with the results.

Study Population: Our study population for the main analysis was based on 10,062 women diagnosed with epithelial ovarian cancer across Finland between 1995 and 2015. We just received information on family history and parity on all individuals which we plan to incorporate. Our observation period was from the date of first ovarian cancer diagnosis to the death date or admin. Censoring data of Dec 31st, 2015. We found 5714 women died and 4348 women who were either alive or had emigrated.

Our initial analysis examined ever/never statin. An ever user was an individual who had even one date of statin prescription. We then incorporated timing of when the first statin use data was available. We also classified statin users as pre-diagnostic if they took statins prior to diagnosis. There are 961 women classified as current users, 1660 women were classified as pre-diagnostic statin users and 7441 women classified as never statin users.

Key baseline characteristics were then compared between women classified as Ever vs Never users. Variables of interest that were considered included, age at diagnosis, follow up time, death (our outcome of interest), stage at diagnosis, cancer subtype, other prescription drug use, surgery, and chemotherapy. All continuous variables were compared using t-tests and categorical variables were tested using Chi-square tests. Overall, ever statin users were older at diagnosis, had longer follow up time, and were less likely to die. Women who had any evidence of Statin use were less likely to have advance stage of cancer at diagnosis. However rates of cancer subtypes were similar between EVER vs NEVER users. Ever users were also more likely to use OTHER prescription cholesterol meds, beta blockers, cardiac meds, and diabetes. Rates of surgery, and chemotherapy were similar in both groups.

We carried out a similar comparison of baseline characteristics for statin use within our observation window. Table 1a and b shows the characteristics of pre-diagnostic and post diagnostic statin versus never users. We found current statin users were younger at age of diagnosis, had longer median follow up time, had fewer deaths, and more likely to have local/regional stage at diagnosis than patients who initiated statin use prior to diagnosis or never took statins. Rates of subtypes were different between the groups. Current statin users

were less likely to use beta blockers, other cholesterol meds, cardiac meds, and diabetic meds than Current statin users were also more likely to have curative surgery and equally likely to have chemotherapy as non-statin users or pre-diagnostic statin users.

Table 1a. Baseline characteristics of all ovarian cancer cases stratified by Never Users, Statin Use initiated prior to diagnosis.

| | OVERAL L | NEVER USED Statin | STATIN USE PRE_DX | CURRENT STATIN USER | P-VALUE |
|----------------------------------|-------------|-------------------|-------------------|---------------------|---------|
| n (%) | 10062 | 7441 | 1660 | 961 | |
| Median age at diagnosis (yrs) | 63 (53-74) | 62 (51-73) | 70 (63-77) | 59 (51-67) | |
| Years of follow-up (median; IQR) | 4 (1.5-9.3) | 3.6 (1.2-8.3) | 3 (1.1-6.0) | 13 (9-17) | |
| Deaths; n | 5714 | 4512 | 990 | 212 | <0.0001 |
| Ovarian cancer deaths | 4218 | 3427 | 698 | 93 | <0.0001 |
| Stage at diagnosis: | | | | | |
| Local/Regional | 3146 | 2206 | 393 | 547 | <0.0001 |
| Advanced/Meta | 5853 | 4461 | 1089 | 303 | <0.0001 |
| Subtypes | | | | | |
| Serous | 4120 | 3003 | 804 | 313 | <0.0001 |
| Endometriod | 1331 | 1015 | 187 | 129 | 0.03 |
| Clear Cell | 324 | 234 | 46 | 44 | 0.03 |
| Mucinous | 932 | 703 | 95 | 128 | <0.001 |
| Borderline | 1904 | 1389 | 211 | 304 | <0.0001 |
| Other | 1451 | 1091 | 317 | 43 | <0.0001 |
| Cholesterol Meds Use | 239 | 55 | 133 | 51 | <0.0001 |
| Beta Blocker Use | 4330 | 2568 | 1150 | 612 | <0.0001 |
| Other Cardiology | 6860 | 4504 | 1506 | 850 | <0.0001 |

| | | | | | |
|---------------|------|------|-----|-----|---------|
| Meds Use | | | | | |
| Diabetes | 1343 | 577 | 478 | 288 | <0.0001 |
| No Surgery | 1219 | 981 | 225 | 13 | <0.0001 |
| Curative | 3490 | 2491 | 463 | 536 | <0.0001 |
| Palliative | 1812 | 1392 | 338 | 82 | <0.0001 |
| Other Surgery | 1568 | 1131 | 267 | 170 | 0.0028 |
| Chemotherapy | 4478 | 3296 | 743 | 439 | 0.0044 |

Table 1b compares all key baseline characteristics by death status including our main exposures of interest. We found that Current statin users and statin ever users were more likely to be alive than those who never used statin. Those who are alive at the end of our study were more likely to have local/regional stage at diagnosis. All subtypes rates were lower among those who were alive. Rates of curative surgery was higher among those who were alive at the end of the study. Whereas the rates of palliative surgery and chemotherapy were lower among those who were alive at the end of our study.

Table 1b. Baseline characteristics of all ovarian cancer by death status

| | OVERALL | DEAD N | DEAD % | ALIVE N | ALIVE % | P-VALUE |
|-------------------------------------|---------|------------------|-----------|---------------|------------|---------|
| n (%) | 10062 | 5714 | | 4348 | | |
| Median age at diagnosis (yrs) | | 70 (60-78) | NA | 56 (46-65) | NA | |
| Years of follow-up (median; IQR) | | 1.8 (0.5-4.0) | NA | 9.4 (5-14) | NA | |
| STATIN_IN OBSERVATION WINDOW | | | | | | <0.0001 |
| CURRENT | 961 | 212 | 22% | 749 | 73% | |
| FORMER | 1660 | 990 | 60% | 670 | 40% | |
| NEVER | 7441 | 4512 | 61% | 2929 | 39% | |
| STATIN_EVERNEVER | 2621 | 1202 | 46% | 1419 | 54% | <0.001 |
| Stage at diagnosis: | | | | | | <0.0001 |

| | | | | | | |
|----------------------------|------|------|-----|------|-----|---------|
| Local/Regional | 3146 | 664 | 21% | 2482 | 79% | |
| Advanced/ | 5853 | 4545 | 78% | 1308 | 22% | |
| SUBTYPE | | | | | | |
| Serous | 4120 | 2771 | 67% | 1349 | 33% | <0.0001 |
| Endometriod | 1331 | 878 | 66% | 453 | 34% | <0.0001 |
| Clear Cell | 324 | 138 | 43% | 186 | 57% | <0.0001 |
| Mucinous | 932 | 395 | 42% | 537 | 58% | <0.0001 |
| Borderline | 1904 | 290 | 15% | 1614 | 85% | <0.0001 |
| Other | 1451 | 1242 | 86% | 209 | 14% | <0.0001 |
| Other Cholesterol Meds Use | 239 | 101 | 42% | 138 | 58% | <0.0001 |
| Beta Blocker Use | 4330 | 2526 | 58% | 1804 | 42% | 0.0064 |
| Cardiology Meds | 6860 | 4124 | 60% | 2736 | 40% | <0.0001 |
| Diabetes | 1343 | 732 | 55% | 611 | 46% | 0.07 |
| No Surgery | 1219 | 1178 | 97% | 41 | 3% | <0.0001 |
| Curative | 3490 | 1212 | 35% | 2278 | 65% | <0.0001 |
| Palliative | 1812 | 1560 | 86% | 252 | 14% | <0.0001 |
| Other_Surgery | 1568 | 842 | 54% | 726 | 46% | <0.0001 |
| Chemotherapy | 4478 | 3026 | 68% | 1452 | 32% | <0.0001 |

Primary Analysis

Statin prescription use is related to heart related issues. In lieu of cholesterol data, we use cardiac meds and medications associated with related co-morbidities to ascertain the predicted probability of statin use. The predicted probabilities are then used as weights to create a balanced sample thereby mitigating any issues of bias due to time varying confounding. Specifically, we use two logistic regression models with statin use as the outcome and then output predicted probabilities. The first logistic regression model includes only time invariant predictors of statin use. The second model uses time varying predictors of statin use including era (1995-2000, 2001-2005, 2006-2010, 2011-2015), era updated cholesterol med use, and all the other time fixed covariates of interest. We then took the ratio of the predicted probabilities from each model and created a person-era specific weight. These weights are then applied to a time-varying cox proportional hazards regression model. The models were then adjusted for age at diagnosis, statin use, stage at diagnosis, subtype, medication use, surgery type, and chemotherapy. The time fixed covariates of the logistic regression models included beta blocker use, cardio med use, diabetic med use, age at diagnosis, stage, and subtype. The time

varying covariates include whether patient was on cholesterol medication at the start of each era using the prescription date. The time varying models also included calendar time and era. The weighted cox model were adjusted for time, age at diagnosis, statin use, stage at diagnosis, surgery, and chemotherapy. Table 2a compare current statin use to no statin use on mortality (sample size is 8402 due to the exclusion of former users in this comparison) and Table 2b compares the effect of former statin use to no statin use on mortality (sample size is 9101 due to the exclusion of current users in this analysis)

Table 2a. Association of Current Statin Users versus never users based on time varying marginal structural models N=8402)

| | Unweighted Survival Model HR (95% CL) | Logistic regression model for Statin use OR (95% CL) | Weighted Survival Model: HR (95% CL) |
|------------------------|---|---|--|
| Age at diagnosis (yrs) | 1.043 (1.04-1.046) | | 1.042 (1.039-1.045) |
| Statin User_ | 0.29 (0.25-0.34) | | 0.65 (0.57-0.74) |
| Stage at diagnosis: | | | |
| Local/Regional n (%) | REF | | REF |
| Advanced/Meta | 2.78 (2.52-3.08) | | 3.16 (2.81-3.55) |
| Subtype | | | |
| Serous | 2.09 (1.77-2.46) | | |
| Endomet | 2.35 (1.97-2.80) | | |
| Clear Cell | 2.25 (1.77-2.86) | | |
| Mucinous | 2.49 (2.08-2.98) | | |

| | | | |
|----------------------|---------------------|---------------------|-----------------------|
| Borderline | REF | | |
| Other | 2.68 (2.24-3.20) | | |
| Cholesterol Meds Use | 1.05 (0.77-1.43) | 4.35 (2.67-7.08) | |
| Beta Blocker Use | 0.87 (0.80-0.94) | 1.62 (1.35-1.95) | |
| Cardio 2 Meds Use | 0.84 (0.78-0.92) | 3.41 (2.65-4.39) | |
| Diabetes Meds Use | 1.06 (0.95-1.18) | 3.93 (3.24-4.78) | |
| No_Surgery | REF | | REF |
| Curative | 0.18 (0.17-0.20) | | 0.18 (0.156-0.207) |
| Palliative | 0.41 (0.37-0.45) | | 0.41 (0.356-0.463) |
| Other_Surgery | 0.25 (0.22-0.27) | | 0.25 (0.21-0.28) |
| Chemotherapy | 0.69 (0.64-0.74) | | 0.69 (0.63-0.76) |

Table 2b. Association between Statin users (who initiated use prior to diagnosis) versus never users based on time varying MSM (N=9101)

| | Unweighted Survival Model HR (95% CL) | Logistic regression model for Statin use OR (95% CL) | Weighted Survival Model: HR (95% CL) (ONLY MEDS) |
|--|---|---|---|
|--|---|---|---|

| | | | |
|------------------------|----------------------|-----------------------|---------------------|
| Age at diagnosis (yrs) | 1.042 (1.04-1.05) | | 1.04 (1.04-1.04) |
| Statin Users | 0.77 (0.71-0.84) | | 0.63 (0.58-0.70) |
| Stage at diagnosis: | | | |
| Local/Regional (%) | REF | | REF |
| Advanced/Meta | 2.78 (2.52-3.08) | | 2.78 (2.49-3.10) |
| Subtype | | | |
| Serous | 2.17 (1.84-2.55) | | |
| Endomet | 2.42 (2.04-2.88) | | |
| Clear Cell | 2.23 (1.76-2.82) | | |
| Mucinous | 2.49 (2.08-2.98) | | |
| Borderline | REF | | |
| Other | 2.86 (2.41-3.40) | | |
| Cholesterol Meds Use | 0.81 (0.64-1.02) | 10.32 (7.27-14.64) | |
| Beta Blocker Use | 0.84 (0.78-0.90) | 2.33 (2.03-2.68) | |
| Cardio 2 Meds Use | 0.87 (0.81-0.94) | 2.87 (2.34-3.51) | |

| | | | |
|-------------------|---------------------|---------------------|---------------------|
| Diabetes Meds Use | 1.10 (0.99-1.21) | 3.21 (2.77-3.71) | |
| No_Surgery | REF | | REF |
| Curative | 0.19 (0.17-0.21) | | 0.20 (0.18-0.22) |
| Palliative | 0.42 (0.38-0.45) | | 0.43 (0.39-0.48) |
| Other_Surgery | 0.25 (0.23-0.28) | | 0.26 (0.23-0.29) |
| Chemotherapy | 0.70 (0.65-0.75) | | 0.69 (0.63-0.74) |

AIM 2:

Aim 2a) assess the anti-tumor effects of lovastatin alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3 and OVCAR5 cells; b) to determine the molecular mechanism by which lovastatin inhibits tumor growth.

Major Task 1: Obtain approvals from Johns Hopkins Animal Care and Use Committee (IACUC) and the DOD Animal Care and Use Review Office (ACURO).

Progress: Completed Year 1

The approvals to conduct the proposed animal studies were obtained.

Major Task 2: In vivo anti-tumor study.

Task 2-1: Establish ovarian cancer cell lines with luciferase-expressing constructs (OVCAR3, SKOV3, & OVCAR5 cells).

Progress: Study completed Year 2

In Fig. 1A, we demonstrated the luciferase activity measured in cultures of ovarian cancer cell lines, OVCAR3-luc, SKOV3-luc, and OVCAR8-luc. All these three cell lines are tumorigenic in athymic *nu/nu* mice and their growth was monitored luciferase activity using a quantitative bioluminescence imaging system (IVIS Lumina imaging) (Fig. 1B).

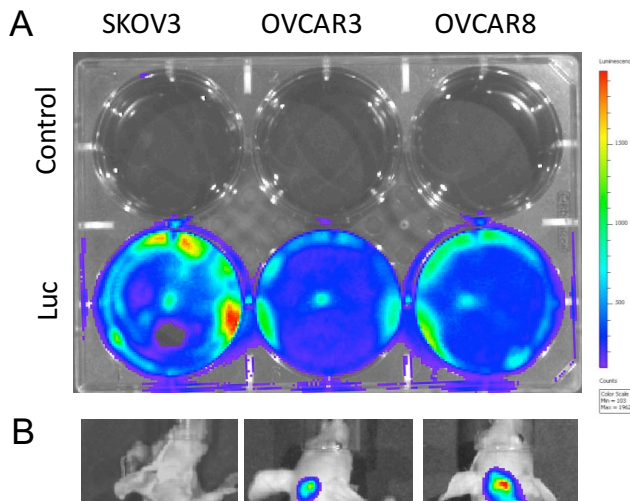


Fig. 1. Establishment of ovarian cancer cells with stable luciferase expression. (A) SKOV3, OVCAR3 and OVCAR8 cancer cell lines were infected with a lentiviral CMV-GFP-T2A-Luciferase vector. Clones with stable expression of luciferase were selected by serial dilution method. Representative images of ovarian cancer cells expressing luciferase or parental control cells. (B) Athymic *nu/nu* mice

To assess drug interactions between lovastatin and paclitaxel and between lovastatin and carboplatin, we measured combination index for both combinations in SKOV3 and OVCAR5 cell lines. Both treatment combinations had a synergistic effect, with $CI < 1$. Both cell lines were more sensitive to the lovastatin/carboplatin combination treatment (Fig. 2).

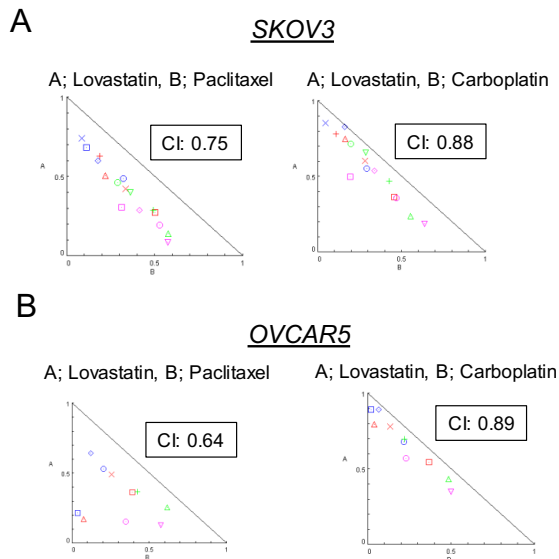


Fig. 2. Combination index plot of lovastatin and paclitaxel and lovastatin and carboplatin in SKOV3 (A) and OVCAR5 (B) cell lines. The Y-axis represents the percent of inhibition for lovastatin, and the X-axis represents the percent of inhibition for either paclitaxel or carboplatin. Combination index is less than 1 for all plots, indicating a synergistic relationship. CI is larger for lovastatin/carboplatin combination in both cell lines, indicating greater sensitivity.

Major Task 3: Conduct

gene knockdown and enzyme inhibitor study to determine which sub pathway(s) is mainly responsible for the anti-tumor effects of lovastatin.

Task 3-1: Purchased siRNAs targeting the enzymes belonging to the three sub pathways in the mevalonate metabolism.

Progress: Completed Year 1

Task 3-2: Purchase small molecule enzyme inhibitors of the mevalonate sub pathways.

Progress: Completed Year 1

Task 3-3: Perform gene knockdown using siRNA in ovarian cancer cell lines (same as described in Task 2). Determine the effects of lovastatin on cellular proliferation, autophagy, and apoptosis as well as the expression of new markers identified in our pilot study.

Progress: Completed Year 1

Task 3-4: Determine the effects of inhibitors on the phenotypes described in Subtask 3 in the same panel of ovarian cancer cell lines. Compare the alterations of phenotypes between 3 and subtask 4.

Progress: Completed Year 1

Major Task 4: Final phase: data analysis, summary, and publication

Task 4-1: Analyze and summarize data to support or refute our hypothesis.

Progress: Completed Year 2

Task 4-2: Prepare manuscript(s) for journal submission.

Progress: Completed Year 2

Aim 2 SUMMARY RESULTS:

Two ovarian cancer mouse models were employed to determine the effect of statins on tumor growth in ovarian cancer. The first one was a genetically engineered model, mogp-TAg, in which the promoter of oviduct glycoprotein-1 was used to drive the expression of SV40 T-antigen in gynecologic tissues. These mice

spontaneously develop serous tubal intraepithelial carcinomas (STICs), which are known as ovarian cancer precursor lesions. The second model was a xenograft tumor model in which human ovarian cancer cells were inoculated into immunocompromised mice. Mice in both models were treated with lovastatin, and effects on tumor growth were monitored. Lovastatin significantly reduced the development of STICs in mogp-TAg mice and inhibited ovarian tumor growth in the mouse xenograft model. Knockdown of prenylation enzymes in the mevalonate pathway (such as PGGT1B and RABGGTB) recapitulated the lovastatin-induced anti-proliferative phenotype, and addition of pathway metabolite geranylgeranyl pyrophosphate (GGPP) reverted the anti-proliferative effects of lovastatin. Transcriptome analysis indicated that lovastatin affected the expression of genes associated with DNA replication, Rho/PLC signaling, glycolysis, and cholesterol biosynthesis pathways, suggesting that statins have pleiotropic effects on tumor cells. Overall, the results suggest that repurposing statin drugs for ovarian cancer may provide a promising strategy to prevent and manage this devastating disease. *Clin Cancer Res.* 2015 Oct 15;21(20):4652-62 (PMID: 26109099) (publication attached in Appendix)

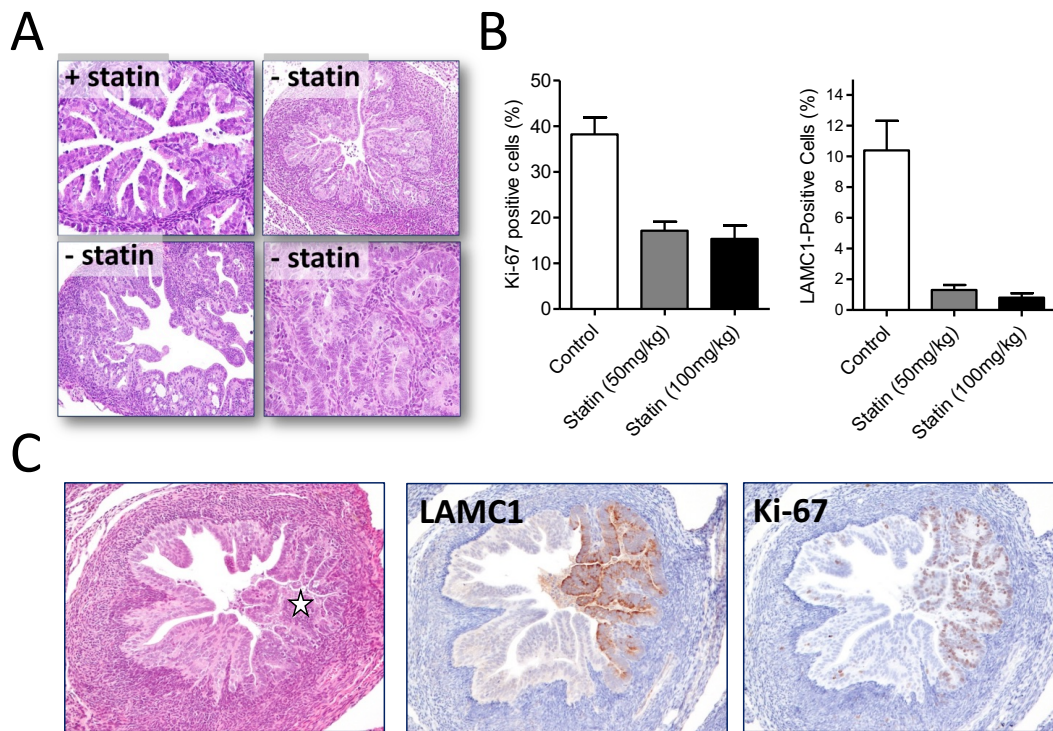


Figure 1. Lovastatin prevents tumor growth in OVGP1-SV40 transgenic mice.

A. Representative section of fallopian tube from OVGP1 mice treated with lovastatin or with vehicle control. **B.** Summary of LAMC-1 and Ki-67 staining. Bar graphs depict percent of LAMC-1-positive or Ki-67-positive epithelial cells among total FT epithelial cells per fallopian tube section. In each experiential group, data were collected from 10 representative sections from each of 10 mice; **p<0.01. **C.** Representative images of H&E, LAMC-1, and Ki-67 staining on tissue sections from fallopian tubes of OVGP1 mice.

C. What opportunities for training and professional development have the project provided?

Aim 1 provided the opportunity for staff and faculty to gain experience in the selection, merging and data management of a large international dataset that included extensive prescription data and inpatient data. By successfully demonstrating the ability to work with these datasets, we recently applied for funding to the DOD include additional Finnish health information databases with regard to a breast cancer proposal.

Aim 2 provided an opportunity for a graduate student, JC Kuan, to obtain knowledge and skills in animal models of ovarian cancer and molecular and biochemical techniques for cancer biology studies. JC is a student in the epidemiology doctoral program, and this project provided him with cross-disciplinary training.

D. How were the results disseminated to the communities of interest?

Dr. Visvanathan plans to present the results from Aim 1 at an upcoming ASCO Annual meeting. In addition she expects to publish the results in a high impact journal and disseminate the data through academic talks at various programs across the Hopkins Campus and other institutions. In addition she will inform ovarian survivors by posting information on a website she oversees for women at high risk for breast and ovarian cancer and mention it at talks she delivers to the lay public on ovarian cancer.

To date there has been one publication from Aim 2 and Dr. Wang presented the results of this research to the Division of Cancer Prevention at the National Cancer Institute (NCI) on 11/17/14. The lecture was entitled "Development of new strategies for ovarian cancer prevention and treatment." Fellow cancer researchers were thereby informed of the potential new treatment and preventative modalities in patients with ovarian cancer.

E. What do you plan to do during the next report period to accomplish the goals?

Not Applicable as this is the conclusion of our NCE. The data however as a result of this completed project provides the foundation for future funding opportunities to apply to study new agents in the mevalonate pathway.

Section IV – IMPACT:

A. What was the impact on development of the principal disciplines(s) of the project?

The results of this study provide robust data on the impact of statin use on ovarian cancer mortality and will inform the discussion of a future clinical trial. The results also demonstrate the importance of using advanced statistical methods when conducting these types of analyses as they can otherwise be misleading.

The successful conduct of this large scale epidemiological study has opened opportunities to merge additional data such as cholesterol levels and CRP levels results to generate even more robust data that could help inform the evaluation of other agents for ovarian cancer patients/

Our publication from Aim 2 provides proof-of-principal evidence for an overarching question: whether persistent intake of statins can protect from gynecologic malignancies, including ovarian cancer. Our work clearly demonstrated that statin intake led to a delay in tumor progression in both genetically engineered mouse and xenograft tumor models of ovarian cancer. The result provides a biological basis for explaining the cancer protection effects of statin intake observed in epidemiology studies. We are now looking at examining lovastatin with some cox based anti-inflammatory therapies.

By combining preclinical exploration and epidemiology in a single grant we have demonstrated a new approach to test other

B. What was the impact on other disciplines?

The successful conduct of this large scale epidemiological study has opened opportunities to merge additional data such as cholesterol levels and CRP levels results to generate even more robust data that could help inform the evaluation of other agents for ovarian cancer patients and other cancers as well as other endpoints such as cardiovascular disease.

Demonstration of the strength of collaborating between two disciplines epidemiology and molecular biology

to address an important clinical question.

C. What was the impact on technology transfer?

No impact on technology transfer.

D. What was the impact on society beyond science and technology?

No impact on society beyond science and technology at this time.

Section V – CHANGES/PROBLEMS:

A. Changes in approach and reasons for change

As mentioned above we decided to use a new complex analytical method after initial analysis of the data in Aim 1 to better account for reasons of statin initiation between the two groups. This had a profound effect on our results.

B. Actual or anticipated problems or delays and actions or plans to resolve

Nothing to Report.

C. Changes that had a significant impact on expenditures

Nothing to Report.

D. Significant changes in use of care of human subjects

Nothing to Report.

E. Significant changes in use of care of vertebrate animals

Nothing to Report.

F. Significant changes in use of biohazards and / or select agents

Nothing to Report.

Section VI – PRODUCTS:

A. Publications, conferences, and presentations

1. Journal publications

Kobayashi Y, Kashima H, Wu RC, Jung JG, Kuan JC, Gu J, Xuan J, Sokoll L, Visvanathan K, Shih leM, Wang TL, Mevalonate Pathway Antagonist Suppresses Formation of Serous Tubal Intraepithelial Carcinoma and Ovarian Carcinoma in Mouse Models. *Clinical Cancer Research*. 2015 Oct15: 21(20): 4652-62 Epub2015 Jun24. PMID: 26109099

2. Books or other non-periodical, one-time publications

Nothing to Report.

3. Other publications, conference papers, and presentations

Abstract to be submitted to ASCO and manuscript in process.

B. Website(s) or other internet site(s)

Nothing to Report at this time.

C. Technologies or techniques

Nothing to Report.

D. Inventions, patent applications, and/or license

Nothing to Report.

E. Other Products

Nothing to Report.

Section VII – PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

A. What individuals have worked on the project?

| | |
|---|---|
| Name: | Kala Visvanathan, MD |
| Project Role: | Principal Investigator |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 1.8 |
| Contribution to Project: | Provided overall project oversight to ensure aims met according to the proposed timeline. Responsible for direct oversight conduct of Aim 1 |
| Funding Support: (other than this award) | None |
| | |
| Name: | Tian-Li Wang, PhD |
| Project Role: | Co-Investigator |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 1.2 |
| Contribution to Project: | Provided oversight for the conduct of Aim 2 |
| Funding Support: (other than this award) | None |
| | |
| Name: | Sharada Modur |
| Project Role: | Co-Investigator |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 1.2 |
| Contribution to Project: | Data analysis for Aim1 |
| Funding Support: (other than this award) | None |
| | |
| Name: | Brenna Hogan |
| Project Role: | Research Data Analyst |
| Research Identifier (e.g. ORCID ID): | NA |

| | |
|---|--|
| Nearest person month worked: | 1.0 |
| Contribution to Project: | Assist with recoding data and data cleaning for Aim1 |
| Funding Support: (other than this award) | None |
| | |
| Name: | Yusuke Kobayash, MD, PhD |
| Project Role: | Post Doc Research Fellow |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 4.8 |
| Contribution to Project: | Performed the experiments proposed in Aim 2 |
| Funding Support: (other than this award) | None |
| | |
| Name: | Teemu Murtola, PhD, MD |
| Project Role: | Finnish Collaborator |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 0 |
| Contribution to Project: | Oversee data acquisition, linkage of registry data and anonymization |
| Funding Support: (other than this award) | At present, not from this grant |
| | |
| Name: | Betty May, MS |
| Project Role: | Research Project Manager |
| Research Identifier (e.g. ORCID ID): | NA |
| Nearest person month worked: | 0.13 |
| Contribution to Project: | Support with IRB, MTA, Data Application, Research & Confidentiality Agreement, and Invoicing |
| Funding Support: (other than this award) | Supported by other grants under Dr. Kala Visvanathan |

B. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Other Support Updates: Kala Visvanathan

ACTIVE (NEW)

Funding Opportunity Number: 90064736 (Visvanathan)

Title: Evaluation of breast cancer susceptibility and prevention strategies in women affected and unaffected with the disease

Effort: 2.0 calendar months (17%)

Supporting Agency: Breast Cancer Research Foundation

Performance Period: 10/01/2016-09/30/2018

Level of Funding:

Project Goals: Compare immune status in BC survivors and cancer free women within a high-risk cohort.

Role: PI

Funding Opportunity Number: N/A (Visvanathan)

Title: Evaluating the Uptake of Screening and Preventative Strategies for Patients at High Risk for Breast Cancer

Time Commitment: 1.20 calendar months (10%)

Supporting Agency: JHMI/Kaiser

Performance Period: 04/01/2017-03/31/2018

Level of Funding:

Project Goals The goal is to evaluate the uptake of screening and preventative strategies for patients with a familial risk at Hopkins and Kaiser, Rockville.

Role: PI

Funding Opportunity Number: 90074212 (Visvanathan)

Title: Impact of Existing or New Co-morbidities among Women Diagnosed with Breast Cancer

Time Commitment: 1.20 calendar months (10%)

Supporting Agency: The Women's Foundation

Performance Period: 04/01/2017-07/31/2018

Level of Funding:

Project Goals: This study will examine whether the number and/or type of co-morbidities at diagnosis is associated with higher mortality and hospitalizations among women diagnosed with both DCIS and invasive breast cancer in Missouri between 2002 and 2012.

Role: PI

Funding Opportunity Number: 7R01CA190428-03 (Epplein/Visvanathan)

Title: Helicobacter pylori protein-specific antibodies and colorectal cancer risk

Effort: 0.60 calendar months (5%)

Supporting Agency: NIH/NCI (sub award only – Duke University)

Performance Period: 04/01/2015 – 03/31/2019

Level of Funding: (subcontract only)

Project Goal: To evaluate the novel association between Helicobacter pylori protein-specific infection and colorectal cancer risk, building the groundwork for significantly strengthening colorectal cancer prevention and screening strategies with a new risk biomarker, as well as the possibility of identifying and exposure that is proven to be modifiable through the use of radiation therapy.

Role: co-Investigator

Funding Opportunity Number: 1R21CA194194-01A1 (Pollack)

Title: The Impact of Provider Social Networks on Breast Cancer Screening

Effort: 0.30 calendar months (2.5%)

Supporting Agency: NIH/NCI

Performance Period: 12/01/2015 – 11/30/2017

Level of Funding:

Project Goal: The Impact of Provider Social Networks on Breast Cancer Screening. Through a national survey of primary care providers and gynecologists, this grant seeks to better understand how physician social networks influence recommendations for breast cancer screening. Parameterized by the survey, we will construct an agent based model to examine ways to alter current patterns.

Role: co-Investigator

PREVIOUS (completed since original submission)

Funding Opportunity Number: W81XWH-11-2-0230 DOD Ovarian Consortium-OC100517 (Kurman/Visvanathan PI project 5)

Title: Prevention of Ovarian High-Grade Serous Carcinoma by Elucidating Its Early Changes

Time Commitment: 2.40 Calendar Months (20%)

Supporting Agency: DOD/ US Army Research Council

Performance Period: 09/30/2011- 09/29/2017

Level of Funding:

Project Goals: The overall objective of the proposal is to utilize morphologic, molecular biologic and molecular pathology techniques and established high-risk cohorts as well as in vitro and mouse models to validate our hypothesis that the precursor of many, if not most, HGSCs begin in the fallopian tube. This will be accomplished through four preclinical studies and one multicenter epidemiologic study.

Role: PI of Project 5

Funding Opportunity Number: 13-A0-00-00066-01

Title: Biomarkers and breast cancer risk prediction in younger women

Effort: 0.12 calendar months (1%)

Supporting Agency: New York University

Performance Period: 09/09/2013-08/31/2016

Level of Funding:

Project Goal: This study will provide robust estimates of the relationship between MIS and breast cancer risk and of the performance of the Gail model 2 with the addition of biomarkers.

Role: co-Investigator

Funding Opportunity Number: SAC110042 (Stearns)

Title: Prospective Studies Correlating Pharmacogenetics Biomarkers and Survival Outcomes

Time Commitment: 0.60 calendar months (5%)

Supporting Agency: Susan G. Komen Breast Cancer Foundation

Performance Period: 07/01/2010-06/30/2016

Level of Funding:

Project Goals: To test for associations between variants in tamoxifen metabolism and estrogen signaling genes, endoxifen concentrations and progression free survival in patients with ER+ metastatic breast cancer treated.

Role: co-Investigator

Funding Opportunity Number: U01CA155340 (Brennan/Johansson/Visvanathan)

Title: One-carbon Metabolism Biomarkers and Lung Cancer Risk

Time Commitment: 0.24 calendar months (2%)

Supporting Agency: NIH/PI organization: International Agency for Research on Cancer (IARC)

Performance Period: 09/23/2011-06/30/2016

Level of Funding:

Project Goals: The overarching objectives of this proposal are two-fold: *i)* to evaluate the OCM pathway in lung cancer etiology with a particular focus on never and former smokers, using both a molecular epidemiology and genetic approach, and *ii)* to measure the role of OCM biomarkers in lung cancer risk prediction in multiple Cohorts.

Role: co-Investigator

Funding Opportunity Number: R01CA160233 (Finkelstein/Visvanathan)

Title: Rare Cancer Genetics Registry (RCGR)

Time Commitment: 0.84 calendar months (7%)

Supporting Agency: NIH

Performance Period: 09/05/2012-06/30/2016

Level of Funding:

Project Goal: To develop a local registry of Johns Hopkins participants with rare cancers who are interested in participating in research studies, along with a specimen bank storing their DNA, and to coordinate the use of these resources in the scientific community of researchers (Coordinating Center is Massachusetts General Hospital).

Role: site PI

Funding Opportunity Number: R01 CA140311 (Umbricht)

Title: Multicenter combined Genetic, Epigenetic and Expression Analysis of DCIS outcome predictors.

Effort: 0.72 calendar months (6%)

Supporting Agency: NIH

Performance Period: 04/01/2011-03/30/2016

Level of Funding:

Project Goal: Identify genetic and epigenetic markers predictive of disease progression among women with DCIS.

Role: co-Investigator

Funding Opportunity Number: R01 CA152071

Title: Circulating Vitamin D Levels and Risk of Breast and Colorectal Cancer: A Pooled Analysis

Effort: 0.60 CM, 5%

Supporting Agency: NIH/NCI

Performance Period: 08/01/2010-07/31/2015

Level of Funding:

Project Goal: We propose to conduct a prospective, pooled analysis of the association between circulating 25-hydroxyvitamin D [25(OH) D] levels and risk of breast and colorectal cancer within a consortium of 15 cohort studies.

Role: co-Investigator, Lead Breast Writing Team

C. What other organizations were involved as partners?

| Organization Name | Location | Contribution to the Project |
|---|----------|---|
| University of Tampere | Finland | <i>Collaboration:</i> Dr. Teemu Murtola is Co-Investigator and responsible for oversight of the data acquisition, linkage of registry data and anonymization and assist Johns Hopkins with the analysis |
| Finnish Cancer Registry | Finland | <i>Other:</i> provide linkage to health information (ovarian cancer case data) for project |
| Social Insurance Institution of Finland | Finland | <i>Other:</i> provide approval for access and linkage of medical and prescriptions reimbursement data for project |

Section VIII – SPECIAL REPORTING REQUIREMENTS:

A. Collaborative Awards: Not applicable

B. Quad Charts: Not applicable

Section IX – APPENDICES:

Attached is copy of publication listed above in Section VI.A.1.

Mevalonate Pathway Antagonist Suppresses Formation of Serous Tubal Intraepithelial Carcinoma and Ovarian Carcinoma in Mouse Models

Yusuke Kobayashi^{1,2,3}, Hiroyasu Kashima^{1,2}, Ren-Chin Wu^{1,2,4}, Jin-Gyoung Jung^{1,2}, Jen-Chun Kuan^{1,2}, Jinghua Gu⁵, Jianhua Xuan⁵, Lori Sokoll^{1,2}, Kala Visvanathan^{2,6}, le-Ming Shih^{1,2,7}, and Tian-Li Wang^{1,2,7}

Abstract

Purpose: Statins are among the most frequently prescribed drugs because of their efficacy and low toxicity in treating hypercholesterolemia. Recently, statins have been reported to inhibit the proliferative activity of cancer cells, especially those with *TP53* mutations. Because *TP53* mutations occur in almost all ovarian high-grade serous carcinoma (HGSC), we determined whether statins suppressed tumor growth in animal models of ovarian cancer.

Experimental Design: Two ovarian cancer mouse models were used. The first one was a genetically engineered model, mogp-TAg, in which the promoter of oviduct glycoprotein-1 was used to drive the expression of SV40 T-antigen in gynecologic tissues. These mice spontaneously developed serous tubal intraepithelial carcinomas (STICs), which are known as ovarian cancer precursor lesions. The second model was a xenograft tumor model in which human ovarian cancer cells were inoculated into immunocompromised mice. Mice in both models

were treated with lovastatin, and effects on tumor growth were monitored. The molecular mechanisms underlying the antitumor effects of lovastatin were also investigated.

Results: Lovastatin significantly reduced the development of STICs in mogp-TAg mice and inhibited ovarian tumor growth in the mouse xenograft model. Knockdown of prenylation enzymes in the mevalonate pathway recapitulated the lovastatin-induced antiproliferative phenotype. Transcriptome analysis indicated that lovastatin affected the expression of genes associated with DNA replication, Rho/PLC signaling, glycolysis, and cholesterol biosynthesis pathways, suggesting that statins have pleiotropic effects on tumor cells.

Conclusions: The above results suggest that repurposing statin drugs for ovarian cancer may provide a promising strategy to prevent and manage this devastating disease. *Clin Cancer Res*; 21(20): 4652–62. ©2015 AACR.

Introduction

The incidence and mortality of epithelial ovarian cancer in the United States has changed very little in the last 20 years; about 22,000 women will receive a new diagnosis this year. Because of the aggressiveness of the disease, once diagnosed, the overall

5-year survival rate is expected to be less than 50%. Part of the problem is that it is difficult to detect ovarian cancer at early stages, and when diagnosed at late stages, there are few effective treatments. Hence, it is critical to develop preventive strategies to reduce the risk of this disease. Currently, for women who are *BRCA* mutation carriers, bilateral salpingo-oophorectomy (BSO) is the recommended surgical procedure to protect these women from developing ovarian cancer. In addition, oral contraceptives, which reduce the frequency of ovulation, have been shown to be effective in reducing the incidence and mortality of ovarian cancer (1). However, neither of these approaches is without concern. Oral contraceptive use is not as safe in older women because the risk of thrombosis increases with age (2); furthermore, its use increases the risk of breast cancer and cervical cancer (3). On the other hand, patients who undergo bilateral salpingo-oophorectomy may suffer from postsurgery complications, especially those symptoms associated with decreased estrogen levels, which include increased adiposity, cardiovascular disease, osteoporosis, and depression at relatively young ages (4–7). Therefore, safer and more cost-effective chemopreventive strategies aimed at preventing or delaying the development of epithelial ovarian cancer are urgently needed.

One potential approach toward chemoprevention of ovarian cancer is to repurpose existing drugs that have been frequently

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Recent studies have led to a paradigm shift in our conceptualization of the cellular origin of ovarian high-grade serous carcinomas (HGSC), the most common and aggressive type of ovarian cancer. It appears that many HGSCs, traditionally classified as ovarian in origin, actually originate from the distal fallopian tube where precursor lesions, serous tubal intraepithelial carcinoma (STIC), can be identified. We used a genetically engineered mouse model that faithfully recapitulates STIC and ovarian tumor progression to determine whether statin intake can prevent the development of STIC. We provide new evidence that lovastatin treatment suppresses STIC development in this mouse model. Furthermore, when applying lovastatin treatment to a xenograft model of ovarian cancer, it efficiently reduces tumor progression. We also elucidate the manifold mechanisms by which statins exert the observed antitumor effects. As statins have been widely prescribed to prevent cardiovascular disease and exhibit low toxicity in patients, our results warrant further investigation to determine the clinical benefit of statins in preventing and treating ovarian cancer.

prescribed to treat non-cancer-related medical conditions in a large population. We can take advantage of the existing population-based database to determine their potency in cancer control. One such class of drugs is statins, which target 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway, which are widely used to prevent and treat hypercholesterolemia. The reasons to focus on statins are multifold. First, it has been recently reported that statins can inhibit the proliferative activity of cancer cells, especially those with *TP53* mutations (8). *TP53* mutations occur in virtually all ovarian high-grade serous carcinoma (HGSC) and in its precursor lesion, serous tubal intraepithelial carcinoma (STIC), suggesting that statins may be effective on HGSC and STIC. Second, statins have been in clinical use for a significant period of time and are well tolerated in patients. Known side effects including alterations in liver function and muscle weakness or tenderness occur only in a small fraction of patients (9). Third, most statins are off-patent generic drugs, offering an inexpensive option as anticancer agents. Fourth, higher circulating levels of low-density lipoprotein (LDL), which can be treated with statins, have been associated with reduced survival among ovarian cancer patients compared with those with LDL levels in the normal range (10). Fifth, statin use is about 11% in the overall U.S. population and as high as 44% in people above 65 years (11). Therefore, there should be collections of existing population-based data to permit researchers to investigate cancer risk or mortality among statin users.

That being said, the data from individual observational studies have been inconsistent and are limited with respect to sample size and lack of detailed information about statin use. The largest study to date included 4,103 epithelial ovarian cancer patients from Danish nationwide registries and observed no difference in ovarian cancer incidence among statin users (12). However, there were only 320 women with HGSC enrolled in this study who were on statins. Contrarily, a recent meta-analysis of 14 studies that included cohort, case-control, and randomized controlled trials,

statin use was associated with a 21% reduction in ovarian cancer risk, and there was no significant heterogeneity among studies (13). To date, only two observational studies (with 150 cases or fewer) have been published that examined the association between statin use and ovarian cancer mortality (14, 15). Both reported a 50% reduction in ovarian cancer mortality among statin users. In a prospective study that examined statin use and mortality from all cancers, a reduction was also observed among women with epithelial ovarian cancer, but the point estimate was not statistically significant (16). Given the discordant results in these reports, it appears important to demonstrate the biologic effects of statins in well-controlled studies such as in animal models of ovarian cancer.

Herein, we determined the antitumor effects of lovastatin, a lipophilic statin, in two animal models of ovarian cancer. The first model is a genetically engineered mouse model, *mogp-TAg*, in which the promoter of oviduct glycoprotein 1 (*OVGP1*) is used to drive expression of the SV40 T antigen in gynecologic tissues (17). These mice spontaneously develop STICs and ovarian/tubal carcinomas at a relatively young age. This transgenic mouse model displays a stepwise progression from normal tubal epithelium to invasive epithelial ovarian cancer, simulating the pathogenesis in humans (18). The second model is a xenograft tumor model in which human ovarian cancer cells are inoculated into immunodeficiency mice. Using the transgenic mouse model, we determined the capacity of lovastatin, as a chemopreventive agent, to suppress the formation of STICs. Using the xenograft model, we assessed the potency of lovastatin in delaying the growth of ovarian tumors. We also explored the molecular mechanisms underlying the antitumor effects of lovastatin.

Materials and Methods

Animal studies

The generation of the *mogp-TAg* transgenic mouse has been described previously (17). Mice were housed and handled according to a protocol approved by the Johns Hopkins University (JHU, Baltimore, MD) Animal Care and Use Committee. The genotype of the *mogp-TAg* transgene was confirmed by tail DNA extraction and polymerase chain reaction (PCR). PCR was performed using the following conditions: denaturation at 94°C for 30 seconds, followed by 30 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 45 seconds, and a final extension at 68°C for 5 minutes. The primer sequences were: forward—GAAATGGAAGATG-GAGTAAA-; reverse—AATAGCAAAGCAAGCAAGAGT-. *mogp-TAg* mice were treated daily with 50 or 100 mg/kg lovastatin diluted in 0.5% methylcellulose by gastric intubation using disposable feeding tubes beginning at 3 weeks of age and continued until euthanasia at 8 weeks. Reproductive tracts were removed, weighed, formalin-fixed, and embedded in paraffin. Because tumor cells occupy approximately 75% of the total mass of the female genital tract in untreated mice, tissue weight was used as an indicator of tumor burden.

To test the therapeutic potential of lovastatin in xenograft tumor models, human ovarian cancer cells, SKOV3-IP or OVCAR5 cells (5×10^6), were injected subcutaneously into the left flank of 6-week-old female mice. The mice were randomly assigned to treatment or control groups; beginning 1 week after tumor cell inoculation, lovastatin (12.5 mg/kg per injection) was administered via intraperitoneal (i.p.) injection twice weekly; atorvastatin (10 mg/kg per injection) was i.p. administered daily (19). Tumor

diameters were measured twice per week using a caliper. Tumor volume (V) was calculated using the formula: $V = A \times B^2/2$ (where A = axial diameter; B = rotational diameter). Excised tumors were homogenized for RNA extraction or were fixed overnight in neutral-buffered formalin and embedded in paraffin blocks.

Cell culture and siRNA transfection

The cell lines used in this study, including SKOV3 and OVCAR5, were purchased from the American Type Culture Collection (ATCC). SKOV3-IP is a derivative line of SKOV3 after three passages in athymic nude mice and is potently tumorigenic. All cell lines were cultured at 37°C, 5% CO₂ in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 U/mL). Cell line authentication was verified by the STR test performed at the Genetic Resources Core Facility at JHU. The STR profiles of SKOV3 and OVCAR5 matched 100% with the published references. The STR profile of SKOV3-IP exhibited 97% match with the SKOV3 profile provided by the ATCC. Lovastatin was used in the *in vitro* experiments. A pilot metabolomics study performed on lovastatin-treated OVCAR3 cell cultures demonstrated that lovastatin potently suppressed the activity of HMG-CoA reductase, resulting in the accumulation of HMG-CoA metabolites in cultured cells.

For gene silencing studies, gene-specific Stealth siRNAs and medium GC control siRNA were purchased from Invitrogen. RNAi duplexes were transfected into ovarian cancer cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Final siRNA concentrations were 50 nmol/L. Viable cells were counted using a T20 automatic cell counter (Bio-Rad).

Western blot analysis

Tumor tissues or cells were homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP40) with Halt Protease Inhibitor Cocktail (1861278, Thermo Fisher Scientific). Protein concentration in tissues or cell lysates was determined with a protein assay kit (Bio-Rad) using bovine serum albumin as a standard. Aliquots of protein lysate (30 µg) were separated by SDS-PAGE, and Western blot analyses were performed using standard procedures. Blots were developed using the Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare UK Ltd.). Primary antibodies used in this study include LC3A (#4599; Cell Signaling Technology, Inc.), LC3B (#3868; Cell Signaling Technology), cleaved caspase-3 (#9664; Cell Signaling Technology), PARP (#5625; Cell Signaling Technology), PCNA (sc-56; Santa Cruz Biotechnology), and GAPDH (#5174; Cell Signaling Technology).

Immunohistochemistry

Paraffin-embedded tissue sections (4 µm) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by incubating tissue sections with Trilogy (Cell Marque; catalog no. CMX833). Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 15 minutes. Sections were preincubated with Dako Antibody Diluent (Dako) at room temperature for 30 minutes, followed by incubation with antibody diluted in Dako Antibody diluent at 4°C overnight. Positive reactions were detected by applying EnVision+/HRP polymer (Dako) for 30 minutes, followed by incubation in DAB substrate for 5 minutes (Liquid DAB+; Dako). The slides were then counterstained with hematoxylin to visualize the cell nuclei. Antibodies used were: human Ki-67 (catalog no. M7240; Dako), mouse Ki-67

(catalog no. 12202; Cell Signaling Technology), LAMC1 (catalog no. HPA001908; Sigma-Aldrich), and phospho-Histone H3 (Ser10) antibody (catalog no. 9701; Cell Signaling Technology).

Quantification of immunohistochemical staining

Ki-67 or LAMC1 positivity in fallopian tubal epithelium of mogp-TAg mice was quantitated as the percentage of positively stained cells. At least 3,200 tubal epithelial cells were counted in each sample.

The proliferative index in xenografts was quantitated as the percentage of Ki-67 positively stained epithelial cells. The total number of epithelial cells and the number of positively stained epithelial cells were counted in each microscopic field (magnification, ×40; Nikon Orthoplan microscope). At least 10 random fields (greater than 2,500 tubal epithelial cells) per experimental group were scored by two independent observers who were blinded to the treatment group. Differences in counts between the observers were <10%.

Microarray analysis

Quality and quantity of total RNA was determined using an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer, respectively. cRNA was synthesized using an Illumina RNA amplification kit (Ambion) following the procedure suggested by the manufacturer. BeadChip hybridization was performed according to the manufacturer's instruction. Arrays were scanned on an Illumina BeadStation 500. BeadChip array data quality control was performed using Illumina BeadStudio software. Probe average intensity signal was calculated with BeadStudio without background correction. Empirical Bayes method (R package limma) was applied to assess the differential expression between DMSO- and statin-treated cells. Differentially expressed probes were defined as having fold change greater than 1.7 and adjusted $P < 0.05$ (false discovery rate).

Microarray data were deposited in the GEO repositories (accession number GSE68986).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on gene expression microarray data using GSEA desktop application v2.0.14 (<http://www.broad.mit.edu/gsea/>) and KEGG gene sets from the Molecular Signature Database (MSigDB) version 4.0 (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>). After ranking genes according to log₂ ratio of expression (control/statin), enrichment scores and significance were calculated by GSEA using 2,000 permutations of each gene set.

Quantitative RT-PCR analysis

RNA was isolated using the RNeasy kit from Qiagen. Total cellular RNA was reverse transcribed into cDNA using an iScript cDNA kit (Bio-Rad). Real-time reverse transcription-PCR (RT-PCR) was performed on a CFX96 iCycler (Bio-Rad) using the SYBR Green I detection method (Invitrogen). Primer sequences are listed in Supplementary Table S1. Relative quantitation of mRNA levels was plotted as fold increases compared with untreated samples. Actin B expression was used for normalization. ΔC_t value (target gene C_t minus Actin B C_t) was averaged from three replicate wells per sample, and $\Delta\Delta C_t$ was calculated as the difference between statin treatment and vehicle control in the same cell line. Relative mRNA quantity was calculated using the $2^{-\Delta\Delta C_t}$ formula.

Analysis of plasma cholesterol and triglycerides

Mice were euthanized at the end of study. Blood was collected by intracardiac aspiration using a 1-mL syringe with a 25-gauge needle and placed in a microcentrifuge tube containing EDTA. Blood was centrifuged, plasma isolated, and cholesterol and triglycerides measured using standard clinical laboratory assays on a Roche Hitachi Cobas c701 analyzer (Roche Diagnostics).

Statistical analysis

Statistical analyses were performed with Prism 5.0 GraphPad software. The Mann–Whitney *U* test was performed to assess tumor volume and immunohistochemical data for LAMC-1, Ki-67, and phospho-Histone H3 for vehicle- and lovastatin-treated groups. Specific analyses performed for each assessment are described in the results and figure legends. In all analyses, data were evaluated using a two-tailed test; $P < 0.05$ was considered statistically significant.

Results

Lovastatin suppresses formation of STICs, precursor lesions of ovarian cancer

To determine whether pharmacologic inhibition of the mevalonate pathway prevents tumor development, we used a genetically engineered mouse model that expresses the SV40 large T antigen driven by the oviduct glycoprotein 1 (*OVGP1*) promoter (mogp-Tag transgenic mice; ref. 17). Mogp-Tag mice consistently develop spontaneous STIC, the precursor lesion of most HGSCs, and uterine stromal sarcoma at 6–7 weeks of age (18). The mice were treated with lovastatin (50 or 100 mg/kg) or control vehicle beginning at 3 weeks of age. The animals were euthanized at 8 weeks to evaluate tumor burden. We found that treatment with lovastatin at either dose (50 or 100 mg/kg) significantly reduced the total tumor mass in the female reproductive tract, as evidenced by the organ weight (Supplementary Fig. S1). Plasma levels of cholesterol and triglycerides were measured at the endpoint, and the data showed significantly reduced cholesterol levels and marginally reduced triglyceride levels in mice treated with lovastatin as compared with controls (Supplementary Fig. S2A).

To determine whether lovastatin reduced the formation and extent of STICs, we compared the histopathology of the fallopian tubes between lovastatin-treated and vehicle-treated mice. Histopathology of fallopian tube section from a representative lovastatin-treated mouse exhibited normal-appearing morphology (top left, Fig. 1A). In contrast, fallopian tube sections from control vehicle-treated mice contained either morphologic features of STICs (top right and bottom left, Fig. 1A) or a tubal carcinoma (bottom right, Fig. 1A). Mice treated with lovastatin exhibited fewer and smaller foci of STICs in individual fallopian tubes than did vehicle-treated mice. At high magnification, STIC from a control mouse could be seen to contain pseudo-stratified epithelial cells with enlarged and atypical nuclei as well as mitoses (right, Fig. 1B), features that were absent in a normal-appearing tube section from a statin-treated mouse (left, Fig. 1B). We also quantified STICs in tissue sections by immunohistochemistry using a STIC-associated marker, laminin C1 (20). We found that the percentage of laminin C1-positive tubal epithelial cells in lovastatin-treated mice was significantly reduced compared with that of the vehicle control group (Fig. 1C and D). Similarly, based on Ki-67 indices, the proliferative activity in STICs of the lova-

statin-treated mice was significantly decreased compared with that of the control group (Fig. 1C and D).

The two different doses of lovastatin used in this study appeared to be safe in animals. The body weights of mice were similar between the two groups (Supplementary Fig. S2A). There was no evidence of lethargy or other physical compromise. Necropsy was performed at the endpoint, and histopathologic examination of internal organs including liver, spleen, kidney, heart, intestine, and brain did not reveal tissue damage in lovastatin-treated mice.

Lovastatin inhibits tumor growth in xenograft models of human ovarian cancer

The studies in the genetically engineered mouse model demonstrated the potency of lovastatin in suppressing spontaneously developing STICs. Next, we determined whether lovastatin exerted antitumor effects on xenograft mouse models of ovarian cancer. SKOV3-IP or OVCAR5 ovarian cancer cells were inoculated subcutaneously into athymic nude mice. Beginning 1 week after tumor inoculation, lovastatin (12.5 mg/kg) or vehicle control was administered by intraperitoneal injection twice a week for 4 weeks. All mice were evaluated for tumor growth twice a week until day 28, when animals were euthanized for endpoint study of tumor burden. The lovastatin treatment was well tolerated by the mice, and there was no effect on body weight measured at the endpoint (Supplementary Fig. S2B and S2C). Blood cholesterol and triglycerides levels were significantly reduced by lovastatin treatment (Mann–Whitney *U* test; Supplementary Fig. S2B and S2C). Furthermore, lovastatin administration significantly reduced the rate of tumor growth in both SKOV3-IP and OVCAR5 tumor xenografts (Fig. 2, left). Immunohistochemistry was performed on the excised tumors using antibodies to Ki-67 (a proliferation marker) and to Ser-11 phosphorylated histone 3 (a mitosis marker). The data showed that tumors from lovastatin-treated animals had significantly fewer proliferating cells than did tumors from vehicle-treated animals (Mann–Whitney *U* test; Fig. 2, middle). The number of mitotic cells was significantly reduced in the lovastatin-treated mice in the OVCAR5 model, while it was marginally reduced in the SKOV3-IP model (Mann–Whitney *U* test; Fig. 2, right).

To determine whether other lipophilic statins exerted an anti-tumor phenotype, we assessed another inhibitor of HMG-CoA reductase, atorvastatin (Brand name: Lipitor), in an OVCAR5 tumor xenograft model. Daily injections of atorvastatin (10 mg/kg) led to significantly reduced tumor sizes as compared with vehicle control treatment (Supplementary Fig. S3A; $P < 0.01$, Mann–Whitney *U* test). Similar to lovastatin, atorvastatin treatment led to reduction in proliferative and mitotic activities as assessed by PCNA and phospho-histone H3 expression levels, respectively, in tumor tissues as compared with vehicle control treatment (Supplementary Fig. S3B).

Effects of lovastatin on autophagy, cellular proliferation, and apoptosis in ovarian cancer cells

We next tested whether statin treatment affected autophagy and apoptosis after lovastatin treatment in SKOV3 and OVCAR5 cell cultures. Cells were incubated with 10 $\mu\text{mol/L}$ lovastatin or vehicle control for 0, 6, 12, 24, 36, or 48 hours, and the expression levels of markers of autophagy and apoptosis were determined by Western blot analysis. LC3A and LC3B are two isoforms

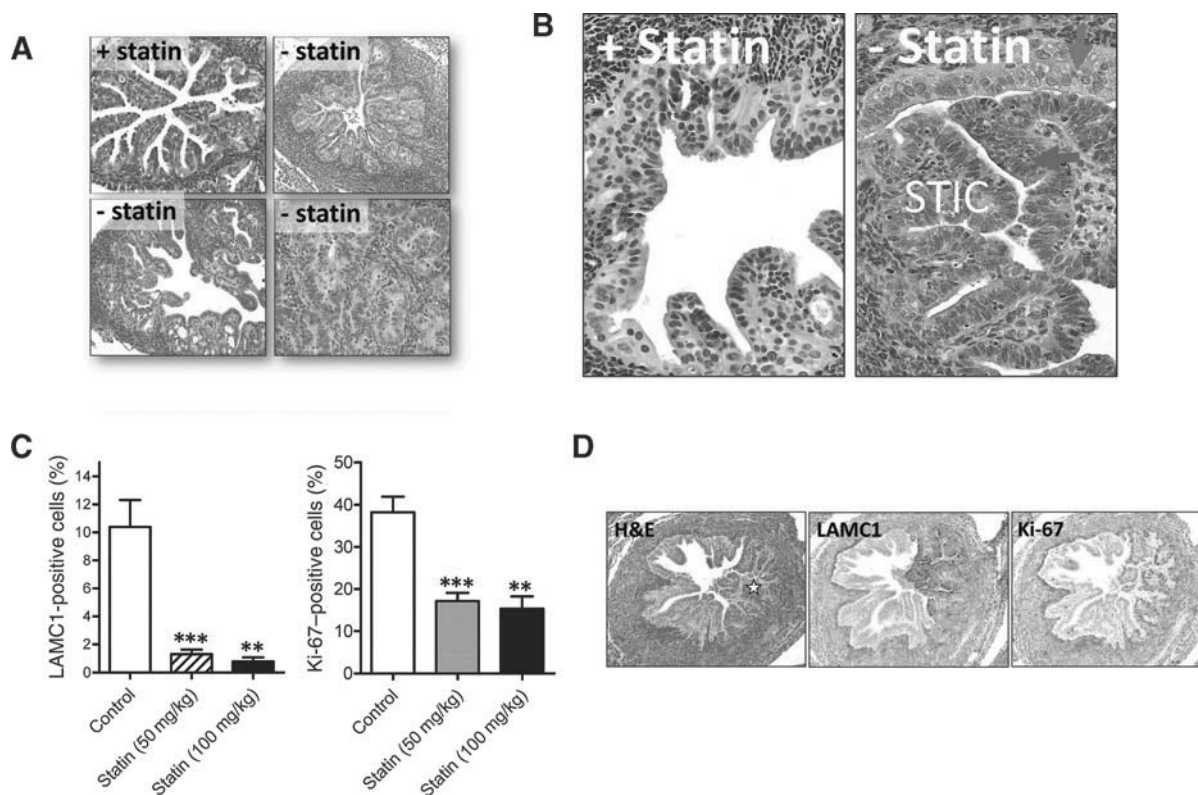


Figure 1.

Lovastatin suppresses the formation of STICs, the precursors of many ovarian HGSCs. A, representative photomicrographs of fallopian tube section from a statin-treated mogp-TAg transgenic mouse showing normal-appearing morphology, whereas extensive STICs are observed in fallopian tube sections from the vehicle-treated mogp-Tag mice. In one of the control mice (bottom right), a tubal carcinoma is also noted. B, higher magnification of fallopian tube sections from statin-treated versus vehicle-treated mice. Red arrow, normal-appearing fallopian tube epithelium; blue arrow, STIC. C, summary of LAMC-1 and Ki-67 staining results. Bar graphs depict the percentage of LAMC-1-positive or Ki-67-positive epithelial cells among total fallopian tube epithelial cells per section. In each experiential group, data were collected from 10 representative sections from each mouse; ***, $P < 0.001$; **, $P < 0.01$, two-tailed Mann-Whitney U test. D, representative images of H&E, LAMC-1, and Ki-67 staining on tissue sections from fallopian tubes of mogp-TAG mice. Star indicates the STICs.

of microtubule-associated protein 1 light chain, LC3, which undergoes posttranslational modification during autophagy. Cleavage of LC3 at the C-terminus yields cytosolic LC3-I. During autophagy, LC3-I is converted to LC3-II through lipidation, which allows LC3 to become associated with autophagosomes. The conversion of LC3-I to faster-migrating LC3-II was used as an indicator of autophagy. Activity of autophagy based on LC3A-II and LC3B-II expression was detected as early as 12 hours after statin exposure, while apoptosis as demonstrated by cleavage of caspase-3 and PARP1 was undetected until 36 hours after statin treatment (Fig. 3A). Next, we performed cell-cycle analysis in lovastatin-treated SKOV3 and OVCAR5 cells using flow cytometry. Lovastatin treatment resulted in a significant, dose-dependent accumulation of ovarian cancer cells in G_0 - G_1 phase, which was accompanied by a concomitant decrease in the number of cells in G_2 -M phase (Fig. 3B). We next attempted to assess autophagy and apoptosis in the tumor xenografts and found that autophagy markers, LC3A-II and LC3B-II, were more abundant in OVCAR5 and SKOV3-IP tumor xenografts in the statin-treated group than in the vehicle-treated group (Fig. 3C). Because increased levels of LC3-I were also observed in statin-treated tumor xenografts, qRT-PCR was performed in these tumors to determine whether LC3 transcript levels were altered by statin treatment. The results showed that both LC3A and LC3B mRNA levels were elevated

in tumors derived from statin-treated mice as compared with tumors excised from control vehicle-treated mice (Mann-Whitney U test; Supplementary Fig. S4). This finding suggests that there is an increased demand of autophagy under statin-induced conditions. As a result, not only was the lipidized LC3 increased, but its transcript and protein levels were also elevated. In contrast, apoptosis appeared to be an inconsistent event in the tumor xenografts because the expression levels of cleaved caspase-3 and cleaved PARP1 varied among different xenografts (data not shown).

Lovastatin affects expression of genes involved in DNA replication, Ras/Rho signaling, and cholesterol biosynthesis

To elucidate the molecular mechanisms leading to the observed antitumor effects of lovastatin, we performed global gene expression analysis using the Illumina Bead Array in ovarian cancer cell cultures that had been treated with 10 $\mu\text{mol/L}$ lovastatin or control vehicle for 48 hours. We observed differential expression of 1,309 genes in OVCAR5 and 4,128 genes in SKOV3 following lovastatin treatment (fold change > 1.7 and FDR < 0.05). Of these, 693 genes overlapped between OVCAR5 and SKOV3 ($P = 1.5 \times 10^{-268}$; hypergeometric test). Ingenuity Pathway Analysis (Qiagen) demonstrated that the most frequently involved canonical pathways included cell-cycle control of DNA

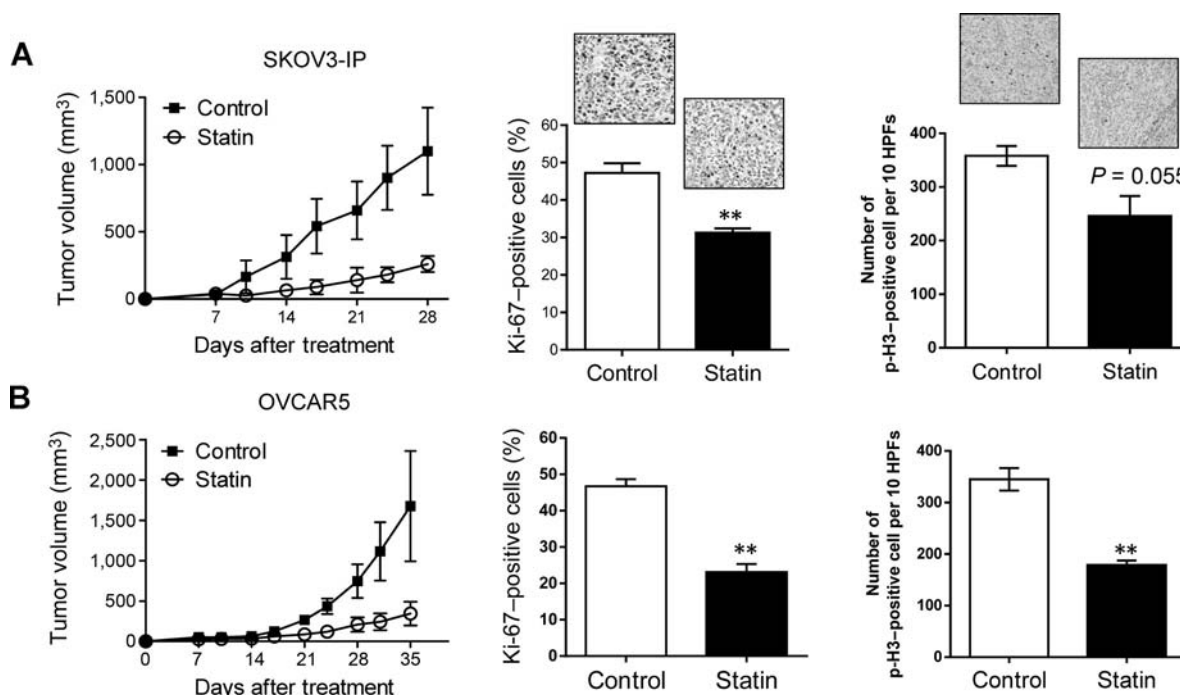


Figure 2.

Lovastatin suppresses growth of human ovarian tumor xenografts. SKOV3-IP (A) or OVCAR5 (B) cells were injected subcutaneously into athymic nude mice. One week after tumor inoculation, mice received i.p. treatment with lovastatin (12.5 mg/kg) twice a week until termination of the study. Tumor volume was measured by a caliper twice per week. The mean tumor volumes are plotted \pm SD ($n = 5$ for each group). Middle, bar graphs depict the percentage of Ki-67-positive cells per high-power field ($\times 400$). Five high-power ($\times 400$) fields were screened per tumor. In total, 25 high-power fields were included for each experimental group of 5 mice. **, $P < 0.01$, two-tailed Mann-Whitney U test. Right, bar graphs depict the average number of phospho-Histone H3-positive cells per high-power field ($\times 400$). Ten high-power ($\times 400$) fields were collected from each tumor, and in total, 50 high-power fields were included for each experimental group. **, $P < 0.01$, two-tailed Mann-Whitney U test. For SKOV3-IP, $P = 0.056$.

replication and phospholipase C (PLC) signaling (Fig. 4A and Table 1). In addition, several members of the Rho and Ras small G protein families are within the PLC signaling pathway. We also performed GSEA to determine the enrichment of KEGG functional pathways in our microarray data. The GSEA results were in agreement with the IPA pathway analysis; again, the gene set involving DNA replication ranked at the top of the list (Supplementary Table S2; Fig. 4B). Interestingly, we observed that several genes in the mevalonate pathway including HMGCS1 and HMGCRC were upregulated in cells treated with lovastatin, suggesting that tumor cells responded to mevalonate pathway blockage by transcriptionally upregulating genes in the same pathway to compensate for the reduced pools of pathway metabolites. Similar regulation of enzymatic activity by transcription in response to metabolite levels in the same pathway is well documented, and is conserved from yeast to mammals (21). Furthermore, a significant fraction of genes in the glycolysis/gluconeogenesis pathway was upregulated in cells treated with lovastatin (Fig. 4C and Supplementary Table S3). Four of the genes including PC, ENO2, ENO3, and HKDC1 in the glycolysis/gluconeogenesis pathway were upregulated in both cell lines following lovastatin treatment (Fig. 4C).

To confirm expression changes induced by lovastatin treatment, we used qRT-PCR to assess mRNA expression of several members in the DNA replication and mevalonate biosynthesis pathways in tumor xenografts as well as in tumors derived from mogp-TAg mice (Fig. 4D). Expression levels of MCM2-7 and

MCM10, which encode minichromosome maintenance (MCM) proteins essential for initiation and elongation of DNA replication, were consistently downregulated in lovastatin-treated tumors (Fig. 4D). In contrast, expression of HMGCS1 and HMGCRC, which, as indicated above, encode enzymes in the mevalonate pathway, were significantly upregulated in lovastatin-treated tumors as compared with tumors from control-treated mice (Fig. 4D).

Protein prenylation mediates the antiproliferative phenotype of lovastatin

To determine whether metabolites in the mevalonate pathway (see Supplementary Fig. S5 for pathway outline), including cholesterol, coenzyme Q10 (CoQ10), geranylgeranyl pyrophosphate (GGPP), or farnesyl pyrophosphate (FPP), could rescue lovastatin-induced antiproliferative effects in ovarian cancer cells, we cotreated OVCAR5 and SKOV3 cells with lovastatin (10 μ mol/L) and individual metabolites. The addition of GGPP significantly reverted the antiproliferative effect of lovastatin (Fig. 5A and B), while applying GGPP or FPP as a single agent did not affect proliferation (Supplementary Fig. S6). In contrast, coinubation of ovarian cancer cells with lovastatin and FPP, water-soluble cholesterol, or CoQ10 had no effect on lovastatin-induced antiproliferative effects (Fig. 5A and B). These data suggest that the antiproliferative effect of lovastatin is likely mediated by depletion of endogenous GGPP pools and is less likely to be related to cholesterol.

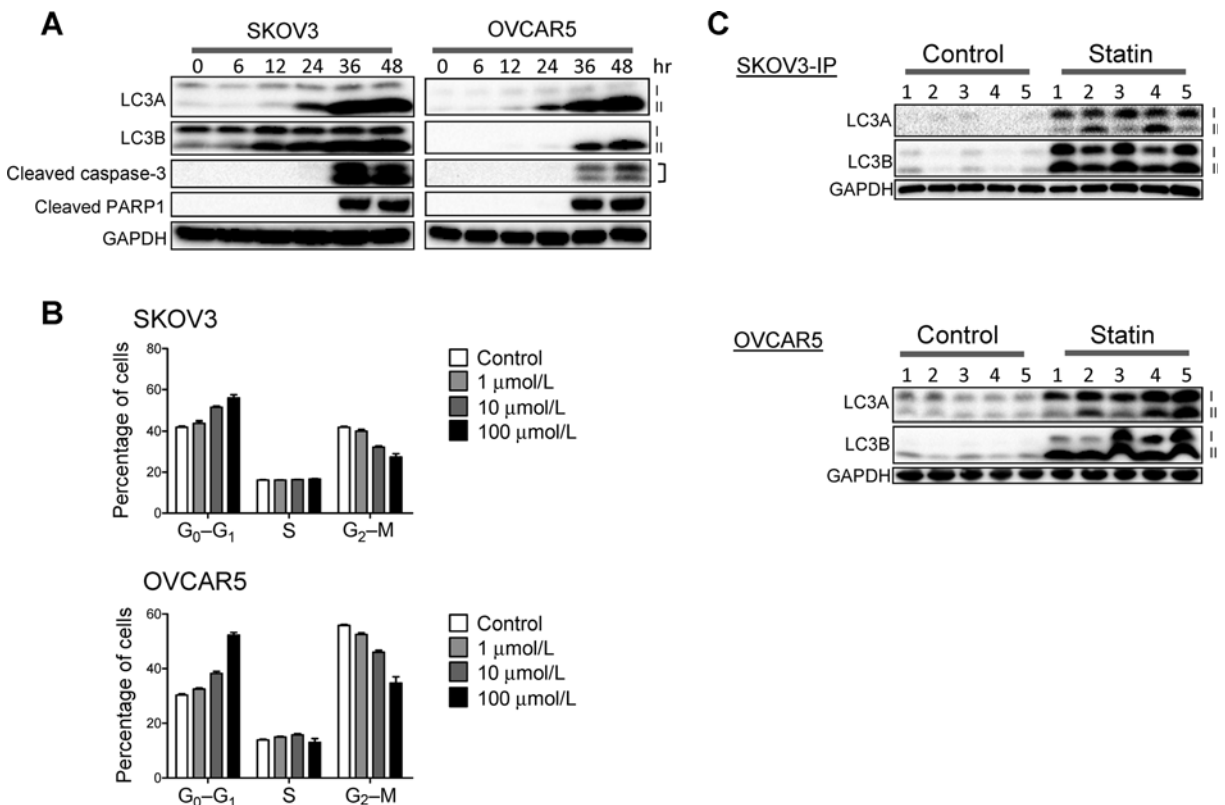


Figure 3. Lovastatin induces autophagy and cell-cycle arrest in ovarian cancer cells. A, SKOV3 and OVCAR5 cell cultures were incubated with lovastatin or vehicle control for various times and were harvested for Western blot analysis to detect autophagy (LC3A and LC3B) and apoptosis (cleaved caspase-3 and PARP-1). I: LC3-I; II: LC3-II; bracket: cleaved caspase-3. B, SKOV3 and OVCAR5 cells were treated with 0, 1, 10, or 100 $\mu\text{mol/L}$ lovastatin for 48 hours. Cell cycle was measured by flow cytometry using propidium iodide (PI) staining. The percentages of cells in G_0 - G_1 , S, and G_2 -M phases are depicted. C, SKOV3-IP and OVCAR5 xenograft tumors from control and lovastatin-treated mice were excised, lysed, and analyzed by Western blotting using antibodies against LC3A and LC3B to detect autophagy. Blots were stripped and reprobed with GAPDH antibody to verify equal protein loading. Each lane represents a different xenograft tumor sample.

Because the above rescue assay indicated that the geranylgeranylation subpathway was involved in the cytotoxic effect of statin, we used an RNAi approach to further dissect key enzymes in this subpathway (see Supplementary Fig. S5 for pathway scheme). The expression of geranylgeranyltransferases, including PGGT1B and RABGGTB, was downregulated in SKOV3 and OVCAR5 cells by two different siRNAs targeting each enzyme. As a negative control, cells were transfected with nontargeting siRNAs. The knockdown efficiency of each target gene was confirmed by qRT-PCR (Supplementary Fig. S7). Squalene synthase (FDFT1), a critical enzyme in the cholesterol synthesis subpathway, was also included as an experimental control. Knockdown of PGGT1B or RABGGTB significantly reduced proliferation, while knockdown of FDFT1 did not have a detectable effect (Fig. 5C and D).

Discussion

Although anticancer actions of statins in both ovarian tumor cell culture and xenograft models have been reported previously (22–24), the chemopreventive potential of statins in spontaneous ovarian/fallopian tube mouse tumor models has not been previously assessed. We report that lovastatin treatment prevents the formation of ovarian cancer precursors—STICs—in mogp-TAg

transgenic mice. In addition, we demonstrate that lovastatin also reduces the tumor volume of ovarian tumor xenografts. As statin drugs have been widely prescribed to prevent cardiovascular disease and exhibit low toxicity in patients, our results warrant further investigation to determine the clinical benefit of statins in preventing ovarian cancer and in treating advanced-stage ovarian cancer.

The antitumor effect of statins is likely mediated by multiple mechanisms. Statins have been reported to modulate local inflammatory responses; when applied to the tumor microenvironment, this mechanism may help control tumor growth (25). In support of this view, bisphosphonate, a mevalonate pathway blocker, has been recently reported to be uptaken by the tumor-associated macrophages in breast cancer tissues with calcification and the drug may specifically target this type of immune cells (26). On the other hand, previous studies have shown that protein modification by geranylgeranylation is critical for the antiproliferative and/or apoptotic activity of statins on tumor cells (8, 27–30). Geranylgeranylation involves the covalent addition of the GGPP lipid to a conserved motif on proteins and is an essential step in controlling membrane localization. For members of the Rho/Rab or phospholipase superfamilies, geranylgeranylation specifies their localization to cellular membranes, a critical step for signaling activation (31). Rho GTPases are closely involved in

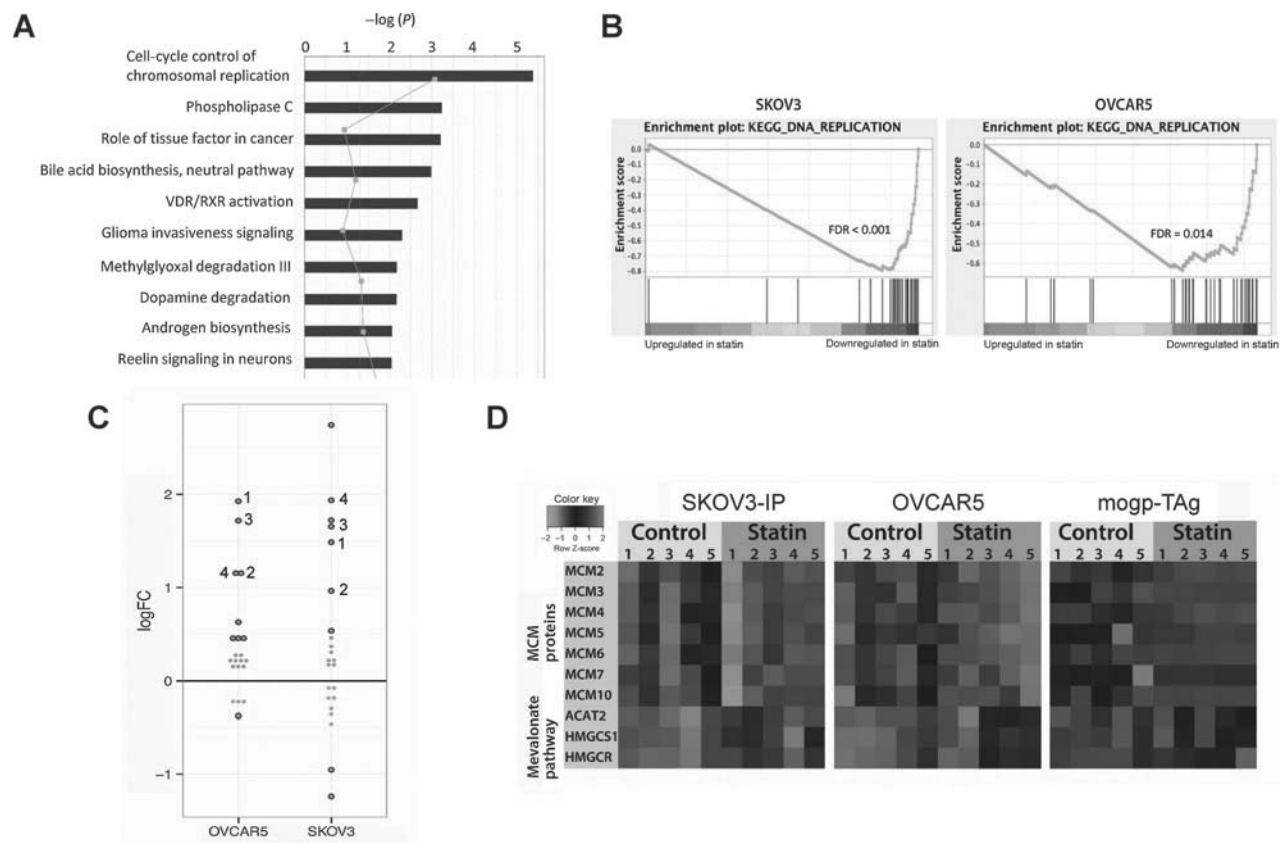


Figure 4. Genome-wide expression profiling of lovastatin-regulated genes using *in vitro* and *in vivo* tumor models. A, canonical pathways of statin-regulated genes revealed by Ingenuity Pathway Analysis. B, lovastatin-regulated genes in SKOV3 and OVCAR5 tumor cells were compared with the KEGG functional pathways to evaluate the enriched gene sets. Shown is the top downregulated gene set, DNA replication. C, expression levels of genes in the glycolysis/gluconeogenesis pathway. Red circle, genes upregulated by lovastatin; blue circle, genes downregulated by lovastatin; circles with black outlines indicate that the differential expressions are statistically significant. Numbers represent genes that are upregulated in both cell lines; 1, 2, 3, and 4 indicate ENO2, ENO3, HKDC1, and PC, respectively. The relative expression values of each data circle can be found in Supplementary Table S3. D, qRT-PCR analysis of expression of genes in DNA replication and sterol biosynthesis pathways in SKOV3-IP and OVCAR5 tumor xenografts and in spontaneous tumors derived from mogp-TAG mice. Normalized expression values derived from three replicates from each sample are shown; green pseudocolor coding represents downregulation, and red coding represents upregulation.

cancer cell morphogenesis, motility, and migration. Rab GTPases control membrane and vesicle trafficking. Phospholipases (PLC, PLD, and PLA) are essential mediators of intracellular signaling and regulate multiple cellular processes that can promote tumorigenesis (32). Given the functional role of geranylgeranylation in

regulating these important signaling pathways in human cancer, inhibition of protein geranylgeranylation is considered a promising target for cancer treatment. Our knockdown study showing that enzymes involved in geranylgeranylation are critical for tumor cell growth further strengthens this view. In addition,

Table 1. IPA pathways regulated by lovastatin in ovarian cancer cells

| Ingenuity canonical pathways | $-\log(P)$ | Ratio | Molecules |
|---|------------|----------|--|
| Cell cycle control of chromosomal replication | 5.38E+00 | 2.35E-01 | MCM5, MCM3, MCM6, MCM2, CDT1, CDC6, ORC6, MCM4 |
| PLC signaling | 3.23E+00 | 7.17E-02 | ARHGEF4, PLD3, RRAS, PLA2G4C, MEF2A, ITGA5, CREB5, HMOX1, PLCB4, RHOB, AHNAK, RHOA, ARHGEF6, LAT, ITPR3, ARHGEF2, ARHGEF3, RHOF, RALGDS, VEGFA, IL8, CTGF, ARRB1, RRAS, ITGA6, IL1B, CSF2, CYR61, ITGB5, MMP1, ITGB3 |
| Role of tissue factor in cancer | 3.21E+00 | 9.23E-02 | |
| Bile acid biosynthesis, neutral pathway | 2.98E+00 | 6.90E-02 | AKR1C1/AKR1C2, AKR1C3, AKR1C4, HSD3B7 |
| VDR/RXR activation | 2.66E+00 | 1.02E-01 | IGFBP6, SPPI, MXD1, CDKN1A, HES1, CEBPB, THBD, CSF2, NCOA3 |
| Glioma invasiveness signaling | 2.30E+00 | 1.06E-01 | RHOB, RRAS, RHOA, PLAU, RHOF, ITGB5, ITGB3 |
| Methylglyoxal degradation III | 2.17E+00 | 1.30E-01 | AKR1C1/AKR1C2, AKR1C3, AKR1C4 |
| Dopamine degradation | 2.16E+00 | 1.05E-01 | ALDH1B1, SULT1A1, SULT1A3/SULT1A4, SMOX |
| Androgen biosynthesis | 2.06E+00 | 1.15E-01 | AKR1C3, AKR1C4, HSD3B7 |
| Reelin signaling in neurons | 2.05E+00 | 9.41E-02 | ARHGEF4, MAPT, ARHGEF6, ITGA6, ITGA5, ARHGEF2, ARHGEF3, ITGB3 |
| IL17A signaling in fibroblasts | 2.04E+00 | 1.25E-01 | TRAF3IP2, NFKBIA, LCN2, CEBPB, MMP1 |
| Estrogen-mediated S-phase entry | 1.95E+00 | 1.43E-01 | CDKN1A, E2F2, SKP2, CDC25A |

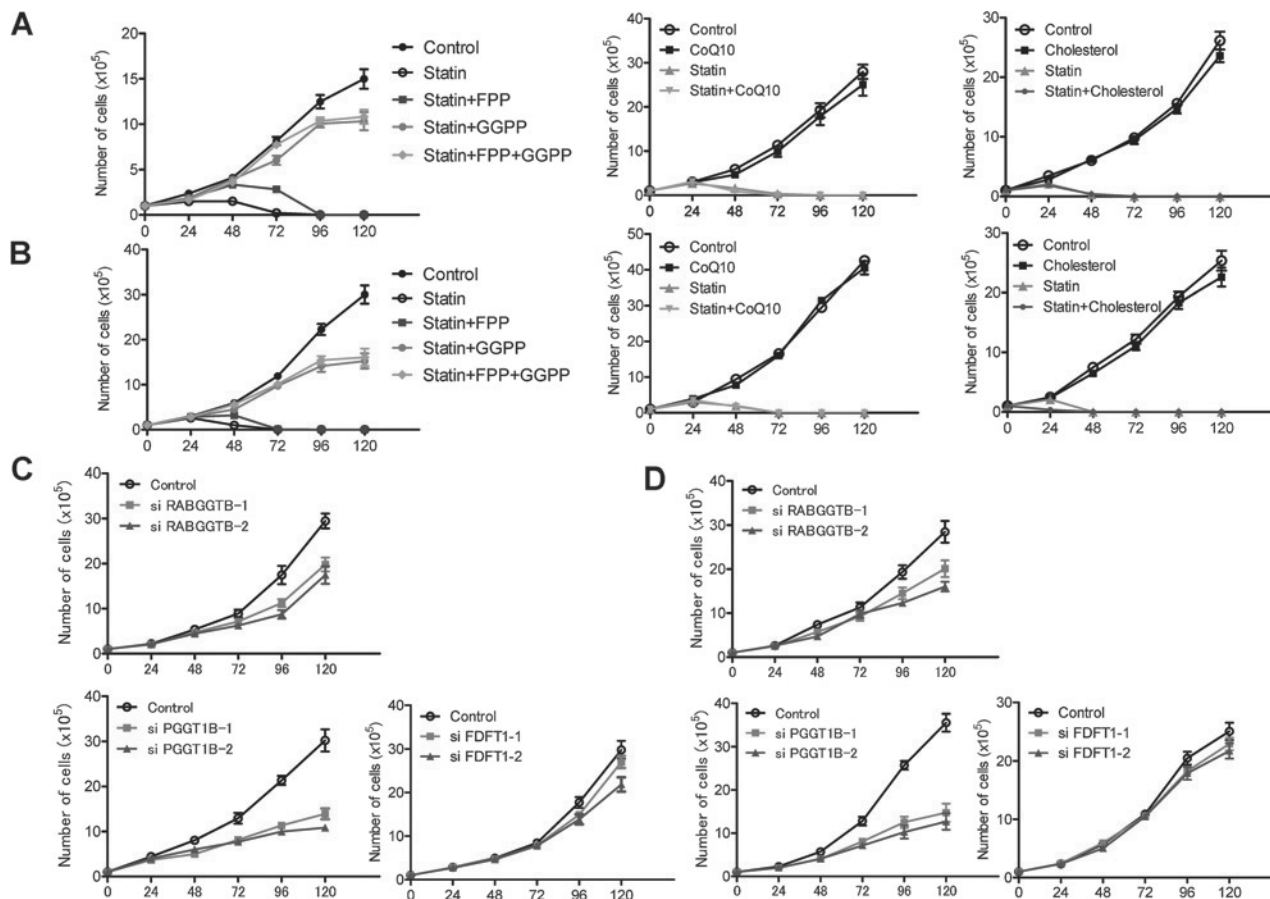


Figure 5.

Exogenous GGPP rescues the antiproliferative effect of lovastatin. SKOV3 (A) and OVCAR5 (B) cells were incubated with GGPP (25 $\mu\text{mol/L}$), FPP (25 $\mu\text{mol/L}$), water-soluble cholesterol (400 $\mu\text{g/mL}$), or CoQ10 (25 $\mu\text{mol/L}$) alone or were coincubated with lovastatin (10 $\mu\text{mol/L}$). To simplify the presentation, data from single-agent GGPP or FPP incubations are plotted separately in Supplementary Fig. S6. Data are presented as mean \pm SD ($n = 3$). C and D, ovarian cancer cell lines, SKOV3 (C) and OVCAR5 (D), were transfected with siRNAs against key enzymes in the geranylgeranylation and squalene synthesis pathways, including PGGT1B, RABGGTB, and FDFT1. Control groups were transfected with nontargeting, medium GC siRNAs. Viable cells were measured at 24-hour intervals over a 120-hour period. Data are presented as the mean \pm SD ($n = 3$).

reducing the amount of GGPP (geranylgeranyl pyrophosphate)—the isoprene lipid precursor of protein prenylation—by statins may compromise Rho/PLC signaling and suppress tumorigenesis.

The pleiotropic effects of statins on tumor suppression were further supported by our transcriptome analysis, which demonstrated that lovastatin-regulated genes participate in a wide spectrum of functional pathways including DNA replication, Rho/PLC signaling, and glycolysis, in addition to participating in cholesterol biosynthesis. The observed upregulation of mRNAs of mevalonate pathway genes such as HMG-CoA reductase is not surprising because negative feedback regulation of transcription in response to the inhibition of HMG-CoA reductase is well documented (33). Another potential antitumor mechanism of statins is suggested by our results demonstrating that statin treatment downregulated genes involved in DNA replication. Most notably, mRNA levels of seven MCM genes were significantly decreased by lovastatin treatment. MCM proteins are known to form replicative helicase complexes, which play a pivotal role not only in DNA initiation and elongation, but also in DNA damage response, transcriptional regulation, and modulation of chromatin structure (34, 35). Thus, statins may directly

or indirectly silence the expression of MCM genes, leading to cell-cycle arrest and accumulation of DNA damage.

Although the doses of lovastatin used in our chemopreventive mogp-TAG model and in the xenograft tumor model were well tolerated and were effective in suppressing tumor growth, they were higher than the doses used for treating hypercholesterolemia in patients. However, the doses used (25–45 mg/kg/day) in a phase I trial is similar to the doses used in this study (36). This prior report found that a dose of 25 mg/kg/day was well tolerated in the enrolled patients and resulted in delayed tumor growth in 1 patient with recurrent high-grade glioma (36). When administering with doses ranging between 25 and 45 mg/kg per day, some patients suffered from myotoxicity, the symptoms of which could be resolved by supplementation with CoQ10 (also called ubiquinone). In future clinical applications, it is most likely that statins will be applied as an adjuvant agent; hence, the dose in the combination setting is expected to be lower than the dose used as a single agent. Nevertheless, future studies are required to determine the maximum-tolerated dose in the combination regimen.

Using mouse models, we observed that lovastatin effectively inhibited tumor growth at both the precursor stage and in

established tumors, but had no noticeable effect on normal tissues. The reasons for the differential effects of statins on normal and neoplastic cells are unclear, but several possibilities can be postulated. First, ovarian tumor cells, as compared with normal cells, may have become more dependent on the mevalonate pathway for sustaining cellular survival and growth. In fact, mevalonate pathway activity is enhanced in many malignancies, including gastric, brain, and breast cancers among others (36–40). In addition, expression levels of HMG-CoA reductase are increased in neoplastic tissues (41, 42). Second, the transcriptional network has been reprogrammed in cancer cells, allowing the mevalonate pathway to control directly or indirectly transcriptional activities of key genes/pathways that collectively promote tumor progression. Although the precise mechanisms remain to be determined, statins may specifically suppress the transcriptional program of tumor cells and, subsequently, affect tumor growth and progression.

Our findings provide critical preclinical data and biologic rationales in evaluating lovastatin for prevention and treatment of ovarian cancer. STIC has been thought to be the precursor lesion in most ovarian HGSC (reviewed in ref. 43), and therapeutic intervention to surgically remove fallopian tubes that may harbor STICs or early cancers has been advocated for reducing ovarian cancer risk, especially for women with predisposing BRCA1/BRCA2 mutations. As a complement to this procedure, this study supports the need to further evaluate the clinical benefit of statins in preventing and treating this devastating disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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