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Computational drug repositioning identifies statins as a modifier of prognostic genetic expression signatures and metastatic behavior in melanoma

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Abstract

Despite advances in melanoma treatment, more than 70% of patients with distant metastasis die within 5 years. Proactive treatment of early melanoma to **prevent** metastasis could save lives and reduce overall healthcare costs. Currently, there are no treatments specifically designed to prevent early melanoma from progressing to metastasis. We used the Connectivity Map (cMap) to conduct an *in silico* drug screen and identified HMGCR inhibitors (statins) as a drug class that might prevent melanoma metastasis. To confirm the *in vitro* effect of statins, RNA-sequencing was

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completed on A375 cells after treatment with fluvastatin to describe changes in the melanoma transcriptome. Statins induced differential expression in genes associated with metastasis and used in commercially available prognostic tests for melanoma metastasis. Finally, we completed a chart review of 475 melanoma patients. Patients taking statins were less likely to have metastasis at the time of melanoma diagnosis in both univariate and multivariate analysis (24.7% taking statins vs 37.6% not taking statins, ARR = 12.9%, p=0.038). These findings suggest that statins might be useful as a treatment to prevent melanoma metastasis. Prospective trials are required to verify our findings and to determine the mechanism of metastasis prevention.

Keywords

metastatic melanoma; fluvastatin; gene expression; computational drug screen

Introduction

Metastasis is the primary driver of cancer mortality.(Dillekås et al., 2019; Zbytek et al., 2008) Despite recent advances in treatment, patients with metastatic melanoma survive on average less than two years after diagnosis.(Kandel et al., 2018; Larkin et al., 2019; Robert et al., 2019) In addition, the cost of treating metastatic disease has increased significantly. (Kandel et al., 2018) Preventing early cutaneous melanomas from progressing to metastasis may decrease healthcare costs and save lives.

Melanomas at high risk of metastasis can be identified by their genetic expressio signature. (Gerami et al., 2015b, 2015a; Kashani-Sabet et al., 2017, 2009; Zager et al., 2018) Retrospective and prospective studies have identified a 28-gene expression signature as an independent predictor of metastasis.(Gerami et al., 2015b, 2015a; Greenhaw et al., 2020; Zager et al., 2018) The immediate clinical utility of these tests is controversial, and the functional role of these genes in metastasis remains elusive. However, the fact that these gene signatures identify melanomas with up to 22-fold higher odds of recurrence or metastasis means they might yield insights into the metastatic process and could even lead to potential therapies.(Chan and Tsao, 2020; Grossman et al., 2020)

The Connectivity Map (cMap) is a publicly available database maintained by the Broad Institute that contains microarray gene expression measurements from over 27,000 pharmaceutical compounds.(Lamb, 2007; Lamb et al., 2006) This database can be queried to identify drugs that induce expression signatures either similar to or opposed to a specified profile. By screening the database for compounds that induce genetic expression directly opposed to a disease signature, the database has been successfully used to identify drugs for computational drug repurposing (the process of discovering new indications for existing drugs).(Chen et al., 2017; Sirota et al., 2011)

We used the cMap to screen for drugs that reverse a high-risk genetic expression profile of melanoma that has been validated in clinical samples.(Gerami et al., 2015a; Greenhaw et al., 2018; Zager et al., 2018) We identified 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) inhibitors (statins) as candidate agents to oppose the high-risk melanoma gene expression profile. Previous studies on statins in melanoma have focused on initiation or

primary prevention and have had mixed results. Two large cardiovascular trials demonstrated reduction in melanoma incidence with statin use, but this effect was not observed in the Women's Health Initiative or two Dutch epidemiologic studies.(Jagtap et al., 2012; Rubins et al., 1999; Splichal et al., 2003) Two meta-analyses also demonstrated no reduction in melanoma incidence with statin use, and a randomized controlled trial of lovastatin for melanoma prevention did not identify any significant decreases in melanocytic atypia or other melanoma initiation markers.(Bonovas et al., 2010; Freeman et al., 2006; Linden et al., 2014) Recently, a Mendelian randomization analysis using the UK Biobank demonstrated that individuals with variants in the HMGCR region, which represent proxies for statin use, had decreased overall cancer risk but did not reach statistical significance for any site-specific cancers.(Carter et al., 2020)

While results have been equivocal in melanoma initiation, there is more consistent evidence that statins may prevent melanoma progression and metastasis. Both *in vitro* and animal models have demonstrated potential mechanisms by which statins could prevent melanoma metastasis through decreasing tumor cell migration, decreasing cell adhesion, and increasing immunogenicity.(Collisson et al., 2003; Kidera et al., 2010; Pich et al., 2013; Zanfardino et al., 2013) One study observed decreased Breslow depth and metastasis rate with statin use, but the decrease in metastasis was not statistically significant in multivariable analysis. (Koomen et al., 2007) Another population based study on all-cause mortality in melanoma patients found a trend towards decreased hazard of death particularly in men, but did not reach statistical significance.(Livingstone et al., 2014)

Here we present an *in silico* drug screen that suggests statins may modify genetic expression correlated with metastasis. We then conduct next generation RNA sequencing to characterize the direct effects of clinically relevant doses of fluvastatin on the melanoma transcriptome *in vitro*. Finally, we explore the association of statin use with metastasis in a retrospective cohort of cutaneous melanoma from our institution.

Results

Identification of statins as a potential treatment for metastasis prevention

We hypothesized that compounds that induced gene expression signatures opposite to that of metastatic melanoma would prevent metastasis. A search of the cMap database identified piroxicam, sotalol, acyclovir, zalcitabine, and simvastatin as potential therapeutic agents (Table 1). The ability of a drug to shift a gene expression signature is measured by its connectivity score (tau), which is a standardized measure ranging from −100 to 100. For each drug in the list of query results, the score corresponds to the fraction of reference gene sets that affect the 28 genes more strongly. The reference gene sets are generated from all reference signatures of drugs in the cMap database. A score of 90 indicates that only 10% of reference gene sets showed stronger effects. In general, a tau of 90 or higher is considered strong and should be considered as hypotheses for further study. Simvastatin had a score of 91. We also checked the score of all statins combined, to ensure that these drugs as a class had a consistent effect. The statin class score was 84.9, which is strong for a class of drugs averaged together. Statins were selected for further study because of their proven long-term

tolerability, benign side effect profile suitable for the intended clinical use as a preventive drug, and possible melanoma chemopreventive effects published in the literature.

Fluvastatin alters the genetic expression profile of melanoma

The cMap data is derived from treatment of cell lines with statins at 10 μM concentrations, which is above the maximal tolerated human dose.(López-Aguilar et al., 1999; Tse et al., 1992) Thus, we sought to characterize the effect of statins on the melanoma transcriptome at clinically tolerable doses. We used RNA-sequencing to measure gene expression of A375 melanoma cells before and after treatment with fluvastatin. The A375 cell line was specifically chosen since it has moderate metastatic potential and has been used in previous mechanistic studies of statins. Fluvastatin was chosen because of its lipophilicity (allowing extrahepatic distribution), benign side effect profile, and excellent bioavailability. We found 2615 differentially expressed genes (Figure 1).

Fluvastatin significantly affected the expression of genes previously shown to be involved in metastasis including *MAGEA1, MAGEA3, MAGEA4, MAGEA6, MAGED1, SOX4, BUB1,* and *KIFC1.*(Brasseur et al., 1995; Jafarnejad et al., 2013; Li et al., 2015; Riker et al., 2008; Weon and Potts, 2015) Genes that drive lymphangiogenesis were also found be significantly decreased by fluvastatin treatment including *FGF2, S1PR5,* and *TGFBRAP1* (Table 2).(Cao et al., 2012; Huang et al., 2013; James et al., 2013)

Consistent with the predicted effect on melanoma metastasis but not initiation, fluvastatin significantly altered the expression of genes included in melanoma prognostic tests (DecisionDx-Melanoma, Castle; Merlin Assay, SkylineDx), but did not alter expression of any genes used in a diagnostic test that distinguishes melanoma from nevi (myPath Melanoma, Myriad Genetics).(Clarke et al., 2015) Genes included in these assays that were significantly shifted are presented in Table 2 (*ITGB3, PLAT, CXCL8, AQP3,* and *KRT14*). This suggests that the effect of statins is specific to progression and metastasis rather than tumor initiation.

Gene ontology (GO) analysis of differentially expressed genes suggested significant enrichment of genes involved in the biological processes of cell proliferation, regulation of cell proliferation, tissue development, response to stimulus, and cell communication (all $p<0.05$). The molecular functions represented included signaling receptor activity, molecular transducer activity, receptor ligand activity, receptor regulator activity, and transmembrane receptor protein serine/threonine kinase binding (all $p<0.05$). Lastly, the cellular components represented included plasma membrane, cell periphery, extracellular matrix, plasma membrane part, and extracellular region (all $p<0.05$).

Patients taking statins have significantly lower incidence of metastasis at diagnosis

To evaluate the clinical impact of statin use on melanoma metastasis, a retrospective cohort of 475 patients with melanoma were reviewed, of which 311 patients met inclusion criteria. The mean age was 64.7 years. The mean Breslow depth in patients taking statins was 3.32 mm, as compared to 2.48 mm in those not taking statins ($p=0.038$) (Table 3). Regional or distant metastasis (defined as a positive completion lymph node dissection or distant metastasis detected on imaging) was identified at diagnosis in 24.7% of patients taking

statins and 37.6% of patients not taking statins (p=0.038). This result was significant in multivariate analysis, after controlling for age, Breslow depth, ulceration, and mitotic rate (p=0.016) (Table 4).

Discussion

Computational prediction has been successful in the past for identifying repurposing opportunities.(Chen et al., 2017; Menden et al., 2019; Sirota et al., 2011) Here we used an *in silico* screen to identify FDA-approved drugs that induce a genetic profile opposed to a validated gene expression profile that predicts melanoma metastasis. We selected statins for further investigation based on their long record of safety, their benign side effect profile consistent with use as a preventive drug, and the literature suggestive of their potential activity in melanoma chemoprevention. Since the *in silico* screen uses data from experiments at doses above the maximal tolerated human serum concentrations, we proceeded to verify the efficacy of statins in appropriately shifting the melanoma transcriptome at clinically achievable doses (3 μM).

We found that fluvastatin caused significant changes in the melanoma transcriptome and affected genes specific to melanoma metastasis at doses below the maximally tolerated dose. (López-Aguilar et al., 1999; Tse et al., 1992) At these doses, fluvastatin did not affect the expression of genes that are used to differentiate nevi from melanoma, consistent with prior clinical trial results demonstrating no effect of statins on the progression of dysplastic nevi to melanoma.(Linden et al., 2014) However, fluvastatin influenced the expression of genes used to measure risk of metastasis in commercially available tests, suggesting that the effect of statins is specific to melanoma progression and metastasis rather than melanoma initiation. This data also implies that history of statin use may be an important factor in interpreting the results of these prognostic tests. Since the drug concentrations we used were lower than those in cMap (therapeutic rather than supratherapeutic) and because we used RNA-seq rather than microarray, there were fewer changes in the 28-gene expression profile than initially predicted by cMap. We found that our RNA-seq validation experiments also revealed changes in genes outside of commercial tests that are known to influence metastatic potential and melanoma development.

Our results suggest potential mechanisms for the effect of statins on melanoma metastasis. Regulation of the G1/S transition appeared to be affected by statin treatment. *CDKN1A (p21)* and *CDKN1C (Kip2)*, which inhibit cell cycle progression in G1 and S phase, are typically suppressed in melanoma, but had significantly increased expression after fluvastatin exposure.(Jalili et al., 2012; Yang et al., 2020) *CUL1 (Cullin 1),* which promotes G1 to S phase transition and drives melanoma proliferation, was significantly decreased after fluvastatin treatment.(Chen and Li, 2010) In addition, increased expression of *CCND3 (Cyclin D3)* has been shown to decrease survival and promote early relapse in melanoma. (Florenes et al., 2000) In our study, we found that *CCND3* expression was significantly decreased after fluvastatin treatment. *KIFC1*, a gene important in centrosome clustering, is overexpressed in primary and uveal melanoma cell lines, as well as in breast and lung cancers.(Pannu et al., 2015) Our study demonstrated that fluvastatin decreased expression of *KIFC1.* Previous studies have demonstrated that metabolic differences in melanoma cells

result in differences in metastatic potential.(Tasdogan et al., 2020) *SLC16A1* (MCT1), which has metabolic functions in lactate transport, has been demonstrated to be an oncogene in malignant melanomas and neuroblastomas and drives melanoma metastasis.(Avitabile et al., 2020) We observed *SLC16A1* downregulation after fluvastatin exposure, but did not achieve statistical significance after correction for multiple hypothesis testing (fold change = 0.797, q=0.062). Lymphangiogenesis is thought to be involved in both metastasis and immune regulation of the tumor microenvironment.(Lane et al., 2018; Lund et al., 2016b, 2016a, 2012) *FGF2, S1PR5,* and *TGFBRAP1* are all involved in lymphangiogenesis and were downregulated by statin treatment. Finally, the *MAGE* gene family has been demonstrated to be expressed in a wide variety of malignancies, including melanoma and is associated with increased invasion and metastasis.(Barrow et al., 2006; Brasseur et al., 1995) We observed decreased expression of *MAGEA1, MAGEA3, MAGEA4*, and *MAGEA6* with fluvastatin treatment.

A prior study found that atorvastatin decreases isoprenylation of RhoC, thereby decreasing migration and invasion in a Matrigel transwell assay of A375 cells, and metastasis in a mouse model.(Collisson et al., 2003) We chose the A375 cell line to build on this prior literature, and our data suggests that statins may also affect lymphangiogenesis, cell cycle regulation, and metabolism to reduce metastasis. The effect on cell cycle regulation identified in this study may be particularly relevant for treatment of familial melanomas induced by *CDKN2A* mutations.(Aspinwall et al., 2008; Goldstein et al., 2007, 2006; Leachman et al., 2009)

We considered the possibility that statins induce gene expression changes that are correlated with metastasis but are not causative of metastasis. If this were true, statin use should not be correlated with the risk of metastasis. Thus, we proceeded to investigate the association between statin use and metastasis in a retrospective cohort of melanoma patients. We found that patients taking statins at the time of biopsy were significantly less likely to have metastasis at the time of melanoma diagnosis as compared to those not taking statins, thereby suggesting that statins may be protective against melanoma metastasis. Statin use remained the strongest independent predictor of metastasis after correction for other prognostic factors including depth, ulceration, mitoses, and age. We considered the possibility that statins may simply be a marker of better access to healthcare resulting in earlier melanoma diagnosis. However, the statin group in our cohort actually had thicker primary melanomas with higher mitotic count indicating later diagnosis. The fact that these patients still had fewer metastases despite significantly worse primary tumors is remarkable.

This study has certain limitations. Our data is retrospective and correlative; despite controlling for all the prognostic factors available, it is possible that there are confounding variables beyond our knowledge such as differences in patient behavior or access to healthcare. We do not have enough follow up data to determine for certain whether patients on statins have better future outcomes, though the reduction in metastases at diagnosis is promising. In addition, our study cohort is from a single tertiary referral center, and thus may be biased towards larger effect size than might be seen in a population based study. Our results need to be confirmed with prospective data and potentially a clinical trial. In addition,

further study of the mechanisms by which statins may affect metastasis is needed to determine causality and may reveal other drug targets.

By leveraging a validated prognostic genetic expression signature, we were able to identify statins as a potential preventive therapy for melanoma metastasis, describe the effects of fluvastatin on the melanoma transcriptome, and demonstrate clinical activity in a retrospective cohort. Since the discovery of statins as a potential prevention for metastasis was based on an existing commercial test, future clinical trials may be able to elegantly select the specific subset of patients who are most likely to benefit. Finally, as other genetic profiles are discovered, this tailored approach may identify additional drugs for prevention or treatment of metastasis.

Methods

In silico selection of candidates for metastasis prevention

The Connectivity Map (cMap) Query Tool (https://clue.io/query) was used to conduct an *in silico* drug screen. The input query consisted of the 28 gene expression profile from a commercially available prognostic test that predicts melanoma metastasis annotated by the desired change in expression (up or down).(Gerami et al., 2015b) Each compound and the corresponding drug class were scored for their ability to oppose the high-risk melanoma gene expression profile using the cMap connectivity score (tau), a standardized measure ranging from −100 to 100. The top 10% of drug candidates were then evaluated for FDA approval status and overall safety profile.

Characterization of transcriptome in human melanoma cells exposed to fluvastatin

We proceeded to characterize the effect of statins on the melanoma transcriptome using a well-established melanoma cell line. Human melanoma cell line A375 (a generous gift of Dr. John Letterio, CWRU) were maintained in standard growth media consisting of RPMI1640 + 10% FBS + 2 mM glutamine and grown in a 5%CO_2 humidified atmosphere at 37°C. Cells were tested bi-annually and shown to be negative for mycoplasma contamination using the Mycoplasma Detection kit (MycoAlert™, Lonza, Basel, Switzerland). For gene expression studies A375 cells were seeded at 2.5E^6 per 10cm^2 dish and allowed to adhere overnight. Test samples (in triplicate) were treated with fluvastatin (purchased from MilliporeSigma, St Louis, Mo) at 3 μM concentration for 24 hours then harvested for RNA extraction using the RNeasy Plus Mini Kit (Cat. #74134, Qiagen, Germantown, MD) as per manufacturer's instructions. Untreated control cells grown side by side were used as the reference control for differential expression analysis. RNA was quantified using the Qubit Broad Range RNA kit (Catalog #Q10210; Thermo Fisher Scientific, Waltham, MA) and diluted to 50ng/ul for RNA sequencing.

Sequencing reads generated from the Illumina HiSeq platform were assessed for quality and trimmed for adapter sequences using TrimGalore! v0.4.2 (Babraham Bioinformatics), a wrapper script for FastQC and cutadapt. Reads that passed quality control were subsequently aligned to the human reference genome (GRCh38) using STAR aligner v2.5.1. Sequence alignment was guided using the GENCODE annotation for hg38. The aligned reads were

analyzed for differential expression using Cufflinks v2.2.1, a RNASeq analysis package which reports the fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. Differential analysis report was generated using the cuffdiff command performed in a pairwise manner for each group. Differential genes were identified using a significance cutoff of q-value < 0.05. The differential expression profiles were then used as input in iPathwayGuide (AdvaitaBio) for pathway analysis.

Multivariate analysis of the effect of statin use on metastasis incidence

To further understand how statins affect melanoma metastasis rates in the clinical setting, we performed a retrospective chart review of patients diagnosed with melanoma in the dermatopathology archive at our tertiary medical center from January 1, 2007 through December 31, 2017. This study was IRB approved. Patients with a histopathological diagnosis of melanoma with Breslow depth greater than 0.8mm or with ulceration were included. Patients with greater than 3 primary melanomas were excluded to avoid confounding by patients with a germline predisposition to melanoma. Data collected included age at diagnosis of primary melanoma; sex; race; immunosuppression status (transplant, HIV, hematologic malignancy); statin use at the time of biopsy; histologic type; Breslow depth; ulceration; mitotic rate; tumor-infiltrating lymphocytes; regression; sentinel lymph node biopsy (SLNB) results; complete lymph node dissection (CLND) results; and presence of metastasis at diagnosis. Univariate and multivariate analyses using logistic regression were performed to determine the relationship of statin use to presence of metastasis at diagnosis, controlled for Breslow depth, ulceration status, and mitotic rate (glm function, R version 4.0.2). A p-value of at least 0.05 was considered significant.

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Data Availability Statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/, hosted at the NCBI Gene Expression Omnibus.

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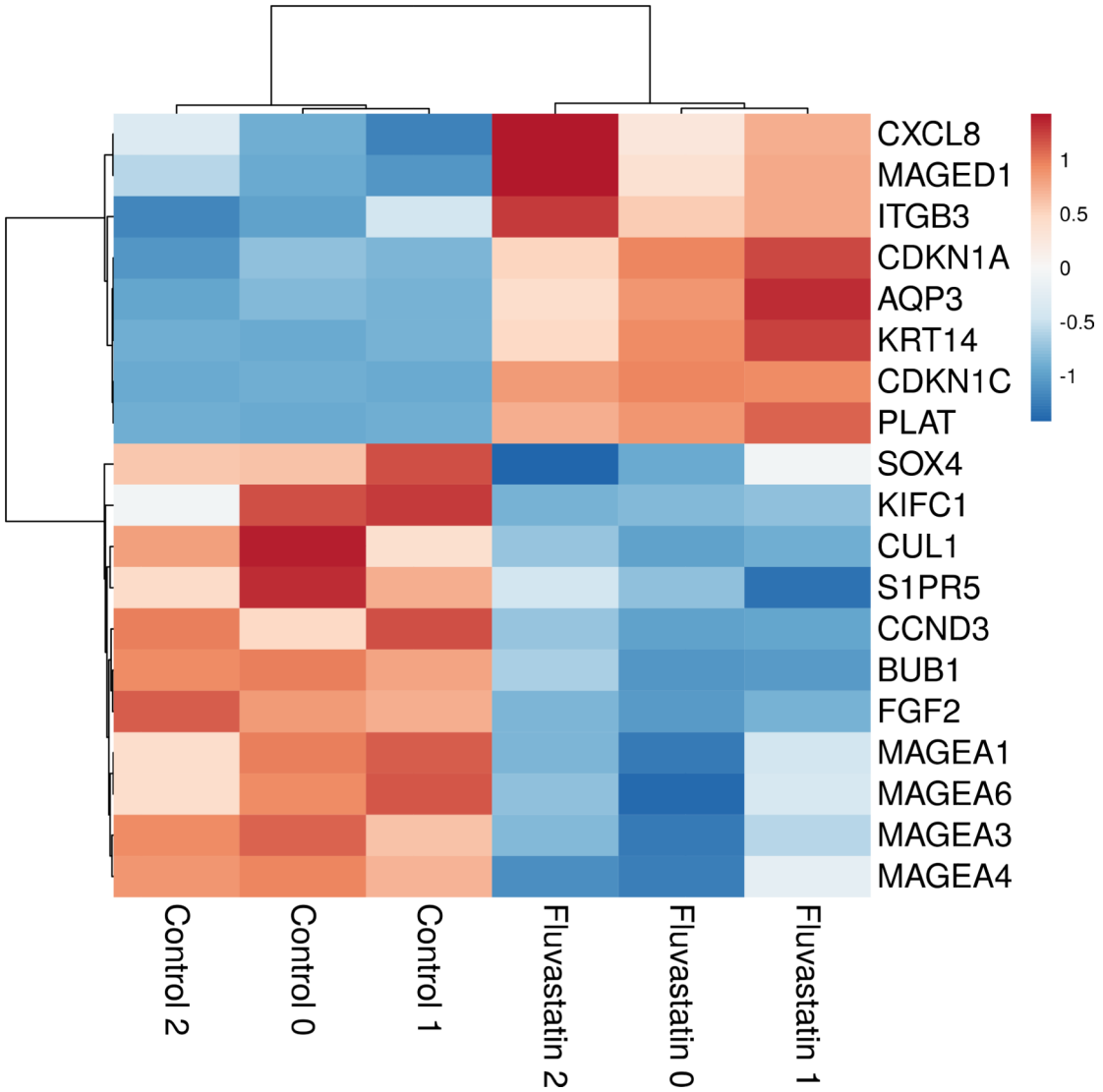

Figure 1.
Heat map of differentially expressed genes related to metastasis.

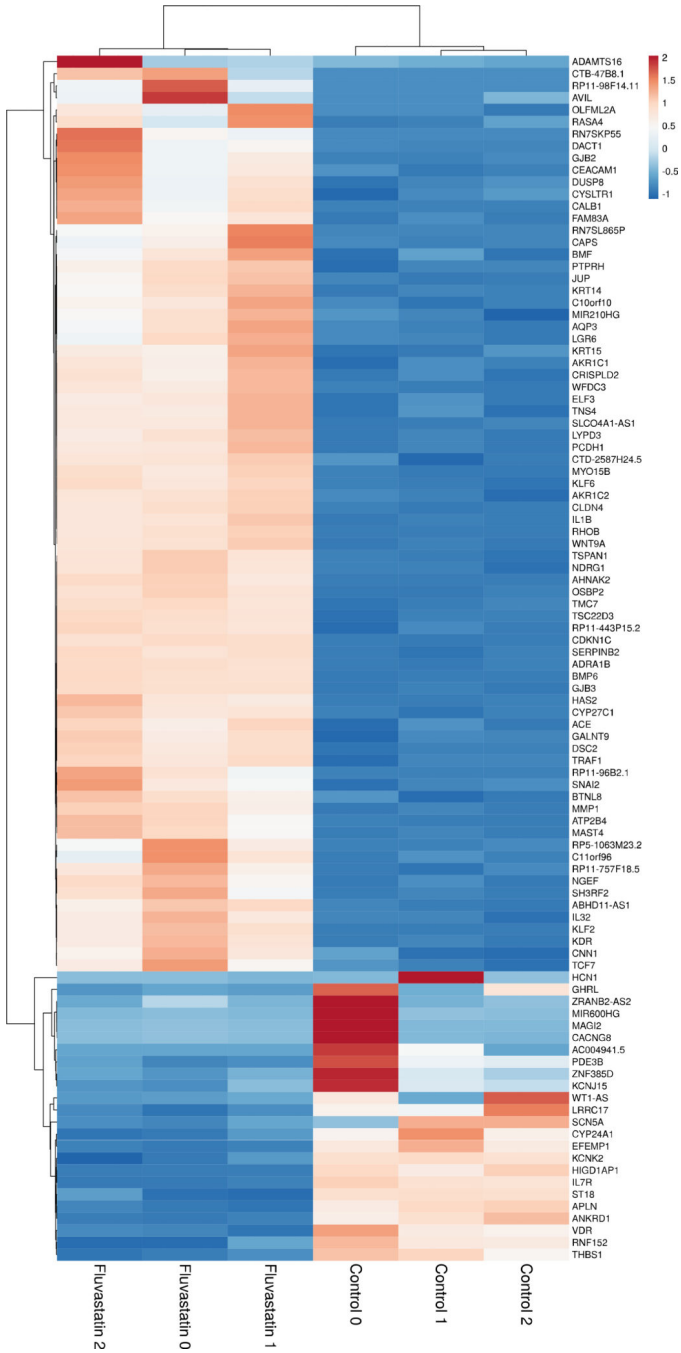

Figure 2.
Heatmap of top 100 differentially expressed genes after fluvastatin treatment.

Table 1.

Candidate Drugs.

Drug	Tau Score	Drug Class	Class Score
Piroxicam	99.93	Cyclooxygenase Inhibitor	−33.86
Sotalol	99.47	Beta-Adrenergic Receptor Antagonist	−13.36
Acyclovir	99.44	DNA Polymerase Inhibitor	23.83
Zalcitabine	99.12	Nucleoside Reverse Transcriptase Inhibitor	97.48
Simvastatin	91.05	HMGCR Inhibitors (Statins)	84.94

Table 2.

Genes of interest differentially expressed after fluvastatin treatment.

Gene symbol	log_2fold change	q-value
MAGEA1	−0.4357	0.0018
MAGEA3	−0.4574	0.0018
MAGEA4	−0.2919	0.0407
MAGEA6	−0.4529	0.0018
MAGED1	0.7222	0.0018
SOX4	−0.4139	0.0018
BUB1	−0.7319	0.0018
KIFC1	−0.4213	0.0355
FGF2	−0.5791	0.0018
S1PR5	−1.3212	0.0292
TGFBRAP1	−0.3389	0.0381
CDKN1A	1.3053	0.0018
CDKN1C	3.2643	0.0018
CUL1	−0.3847	0.0062
CCND3	−0.7194	0.0089
ITGB3[#]	0.5304	0.027
AQP3[*]	3.2162	0.0018
KRT14[*]	2.8530	0.0018
PLAT[#]	1.7389	0.0018
CXCL8[#]	0.8218	0.0018

[*] Castle Biosciences profile

[#] SkylineDx profile

Table 3.

Demographics of patients taking and not taking statin at time of biopsy.

	Taking statin (n=77)	Not taking statin (n=234)	p-value
Mean age (years), n	72.9	61.5	<0.001
Mean Breslow depth (mm), n	3.32	2.48	0.038
Ulceration, n (%)			
Yes	26 (33.8)	79 (33.8)	
No	51 (66.2)	155 (66.2)	0.999
Mitotic Rate (mitoses/mm^2), n (%)			
0	9 (11.7)	28 (11.9)	
1–5	56 (72.7)	181 (77.4)	
6–10	5 (6.5)	21 (9.0)	
>10	7 (9.1)	4 (1.7)	0.015
Metastasis detected during initial workup, n (%)			
Yes	19 (24.7)	88 (37.6)	
No	58 (75.3)	146 (62.4)	0.038

Table 4.

Multivariate analysis for factors that predispose to metastasis at initial workup.

Factor	Odds Ratio (95% CI)	p-value
Age (years)	0.98 (0.97 – 1.00)	0.01
Depth (mm)	1.12 (1.00 – 1.24)	0.04
Ulceration Present	1.44 (0.81 – 2.55)	0.22
Dermal Mitoses (per sq mm)	1.02 (0.95 – 1.10)	0.61
Histologic Type		
Nodular	1	-
Superficial Spreading	0.93 (0.47 – 1.81)	0.82
Lentigo Maligna	0.27 (0.08 – 0.88)	0.03
Other	0.81 (0.39 – 1.67)	0.57
Taking a Statin	0.48 (0.25 – 0.94)	0.03