CHAPTER THREE

STATINS AND THE RISK OF COLORECTAL CANCER IN RELATION TO THE EXPRESSION OF SMAD4

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ABSTRACT

Background

Long-term use of statins is associated with a reduced risk of colorectal cancer but their mechanism of action is not well understood. While they are generally believed to act on *K-ras*, we have previously proposed that they act via influencing the BMP pathway with a critical role for the central pathway element SMAD4.

Methods

Cohorts of statin users and controls were identified using two registries unique to the Netherlands and, if they developed colorectal cancer, their specimens traced. SMAD4 expression was estimated by immunohistochemistry and the mutation status of *K-ras* and *BRAF* assessed from paraffin-embedded colorectal-cancer specimens. We compared the effects of statin use on the relative risk of colorectal cancer in relation to the expression of SMAD4 and the mutation status of *K-ras* and *BRAF*

Results

We identified 68,948 statin users and 94,272 controls that developed 386 and 609 colorectal cancers respectively. Tumors from 325 statin users and 297 controls were analyzed. Statin use conferred a significant reduction in the risk of colorectal cancers that expressed SMAD4 (relative risk, 0.66; 95% CI 0.53 to 0.82), whereas statin use had no influence on tumors with weak or absent expression of SMAD4 (relative risk, 1.02; 95% CI 0.87 to 1.20). There was no relationship between the mutation status of *K-ras* and *BRAF* and reduction in colorectal cancer risk due to statin therapy.

Conclusions

Statin use reduces the risk of colorectal cancers that express SMAD4 but not the risk of colorectal cancers with weak or absent expression of SMAD4.

INTRODUCTION

Statin use is associated with a reduced risk of developing colorectal cancer[1] and a reduced risk of dying from it[2], but this risk reduction varies widely between studies and in meta-analyses is small[3-5]. The mechanism by which statins influence the risk of colorectal cancer is not well understood. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase the rate-limiting step in mevalonate synthesis[6]. Inhibition of the mevalonate pathway not only disrupts cholesterol synthesis but also farnesyl pyrophosphate important for the prenylation of K-ras. Approximately 30% of colorectal cancers have activating *K-ras* mutations[7]. Therefore the current dominant hypothesis for the mechanism of action of statins in colorectal cancer is that they act by inhibiting K-ras thus inhibiting the RAS/RAF pathway[8]. Other mechanisms that are unrelated to K-ras have been proposed including inhibition of angiogenesis[9], inhibition of metastasis and potentiation of the effects of chemotherapeutic agents[10].

We have proposed that statins act on colorectal cancer through activating the bone morphogenetic protein (BMP) pathway. This is based on evidence that statins activate the BMP pathway in bone[11], and on evidence for a crucial role for the BMP pathway in colorectal cancer[12]. In studies *in vitro* and in rodents we have shown that the effects of statins depend on the expression of the central BMP pathway element, SMAD4. Statins have anti-tumor effects on SMAD4-expressing cancer cells, but not on cancer cells lacking SMAD4[13].

If these findings hold true in humans, then statin use should reduce the risk of developing SMAD4 expressing tumors. Similarly, if statins act by inhibition of K-ras then statin use would be expected to alter the likelihood of developing a tumor with *K-ras* or *BRAF* activating mutations. We therefore investigated whether the influence of statin use on the risk of developing colorectal cancer was dependent on the expression of SMAD4 in the tumor, or on the presence of *K-ras* and *BRAF* mutations in a large patient cohort. We first performed a large population-based cohort study to investigate the influence of statin-use on colorectal cancer incidence and subsequently analyzed SMAD4 expression and *K-ras* and *BRAF* mutation status in the tumor specimens.

METHODS

National registries

The PHARMO record linkage system includes a pharmacy database and records anonymously all dispensed drug prescriptions from more than 200 pharmacies in more than 50 regions scattered over the Netherlands, representative for the Netherlands as a whole[14,15]. Currently it covers more than 2 million residents regardless of type of insurance, or 12,5% of the Dutch population. Since patients in the Netherlands register at a single pharmacy

to which they bring all prescriptions from general practitioners or medical specialists, dispensing histories are virtually complete[16]. The computerized drug dispensing histories record the type and quantity of the dispensed drug, prescriber details, dispensing date, and the prescribed daily dose. The Dutch National Pathology Registry (PALGA) contains data from all pathological examinations performed in the Netherlands allowing it to serve as the main database source for the Dutch Cancer Registry[17]. PALGA contains abstracts of the pathology reports consisting of encrypted patient identification and a summary of the report and the diagnosis (SNOMED).

Study Population

Cardiovascular risk is associated with a higher risk of colorectal cancer[18]. To control for this confounder in statin users, the study base included all patients receiving beta-blockers for more than 6 months within the study period defined as between January 1, 2000 and December 31, 2007. Within this study base of patients with a similar risk of cardiovascular disease, we performed a nested cohort study. Patients receiving more than 6 months of statin treatment during the study period were included into the statin user cohort from the date of the first statin prescription or the begin of the study period whichever came last. Patients receiving no statins during the study period were entered into the control cohort. All patients with a history of colorectal cancer, chemotherapy, or radiotherapy before the prospective date of cohort inclusion were excluded. Using the Dutch National Pathology Registry (PALGA) all cases of colorectal cancer occurring within the study period were identified.

Tumor analysis

We retrieved a sample of 622 colorectal cancer pathological specimens, 325 from statin users and 297 from controls. We aimed to retrieve a minimum of 281 specimens from each group guided by a prior power analysis. We collected all available specimens from the largest pathological laboratories geographically spread around the country. Our analysis was limited by the availability of paraffin blocks with sufficient amounts of tumor tissue. All samples were handled according to the medical ethical guidelines established by the Dutch Federation of Medical Sciences. In rectal cancer we analyzed biopsies from the time of diagnosis rather than irradiated resection specimens. The baseline characteristics of patients with colorectal cancer whose tumors we analyzed did not differ from patients whose tumors we did not analyze.

SMAD4 analysis

For SMAD4 immunostaining on whole tissue sections we used a monoclonal antibody against SMAD4 (Clone B8, Santa Cruz Biotechnology, Inc.; 1:400). 4-µm sections were deparaffinized and blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2

in methanol for 20 minutes. Antigen retrieval was performed in Tris-EDTA buffer (10 mmol/L/1 mmol/L, pH 9.0) for 30 minutes at 97°C. Nonspecific binding sites were blocked in PBS with 10% normal goat serum for SMAD4 for 10 minutes. This was followed by 1-hour antibody incubation for SMAD4 at room temperature. Antibody binding was visualized using the Powervision+poly-HRP detection system (ImmunoVision Technologies, Co.) and 3,3-diamino-benzidine (DAB, Sigma). Sections were counterstained with hematoxylin.

Two pathologists both unaware of any data concerning the participants, independently interpreted SMAD4 expression, using a standardized grading system (absent, weak, normal). The pathologists classified staining of tumor cells as "normal" if SMAD4 expression was at the same level of intensity as that in stromal cells; "weak" and "absent" staining indicated progressively decreasing degrees of loss of expression. The concordance between the two pathologists was 87% ($\kappa = 0.70$). If the intensity of immunostaining was normal, tumors were classified as cancers with high SMAD4 expression (SMAD4–high). If immunostaining intensity was weak or absent, tumors were classified as having low SMAD4 expression (SMAD4–low) (Fig. 1).

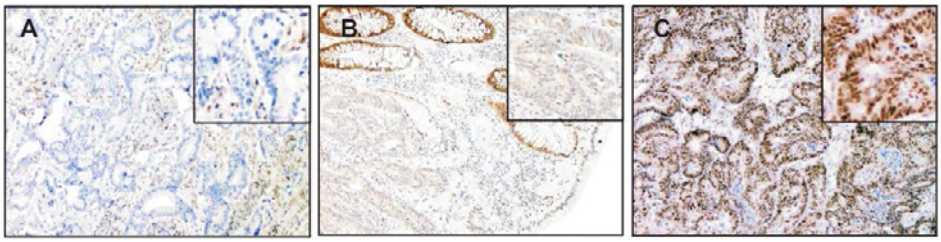

Figure 1. SMAD4 expression in immunohistochemical assays of colorectal cancer tissue and mucosa. In panel A, SMAD 4 is not expressed in carcinoma as compared with normal mucosa. Panel B shows weak expression of SMAD4 in carcinoma as compared with normal mucosa. Panel C shows normal expression of SMAD4 in carcinoma as compared with normal mucosa.

K-ras and *BRAF* mutation analysis

DNA was extracted from formaldehyde-fixed, paraffin-embedded tumor biopsy or resection specimens using NucleoSpin Tissue XS according to the manufacturers protocol. The DNA was screened for *K-ras* mutations in codon 12 and 13 using High-Resolution Melting analysis and subsequent sequencing. BRAF mutation in the specimens was assessed by real-time PCR mutation detection using TaqMan MGB probes. (More detailed description in supplementary data)

Statistical analysis

For the initial analysis of the effect of statin use on unselected colorectal cancer incidence, time-dependent Cox regression, adjusted for age, gender and NSAID use, with time since beta-blocker use as time scale, was used employing the Andersen-Gill method. Statin use

was coded as a time-dependent covariate taking values 0 (no statin use), 1 (statin use in the first six months), 2 (statin use between six and 12 months), and 3 (12 months or more of statin use). Statin use was considered to be cumulative; interrupting use of statin did not reset the value of the time-dependent covariate. The primary analysis compared the values 2 and 3 as defined above together with 0 and 1 together, but sensitivity analyses were conducted comparing different combinations (0,1,2 versus 3). NSAID use and Age were also added as time-dependent covariates. NSAID use was coded as 0 (no current use) or 1 (current use). Age was discretized and updated at the start of every year. Using age as a continuous time-dependent covariate would not have been feasible, given the large sample size. R, version 2.15.0, was used for this analysis.

Cox proportional hazards modeling was used to control for multiple variables simultaneously and to calculate 95% confidence intervals. Multivariate relative risks are adjusted for age, sex, length of follow-up, diabetes, inflammatory bowel disease, adenoma removal and NSAID use. For the analyses restricted to women, the multivariate models additionally adjusted for postmenopausal hormone-replacement therapy. To compare the association between statin use and the risk of colorectal cancer in relation to SMAD4 expression or *K-ras* and *BRAF* mutations in the cancer, we used the Pearson Chi-square test and calculated the odds ratio and relative risk. These outcomes were subsequently extrapolated to the general population. This method permits estimation of separate regression coefficients for statin use stratified according to the type of outcome (e.g., SMAD4-high colorectal cancer vs. SMAD4-low colorectal cancer). We assessed the statistical significance of the difference between the risk estimates according to tumor type by a likelihood-ratio test that compared the model that allowed for separate associations of statins according to SMAD4 expression with a model that assumed a common association. We used SAS software (version 9.2; SAS Institute Inc, Cary, NC) for all analyses. All p values are two-sided.

Power analysis

40% of colorectal cancers have normal expression of SMAD4. If the approximately 10% reduction seen in unselected cancers in meta analyses is exclusively due to reductions in SMAD4 expressing cancers, we would expect this proportion to drop to approximately 30%. With alpha = 0.05 and a beta = 0.8, a sample size of 281 tumors in each group is required.

RESULTS

Among the 696,516 beta-blocker users identified to provide the study base we identified 68,948 statin users and 94,272 controls. The group of statin users included significantly more men and diabetics ($P<2e^{-16}$ for both) than the control group but otherwise the groups showed no differences in age, duration of follow up, inflammatory bowel disease, adenoma

removal, use of NSAIDs and postmenopausal hormones (among women), (P>0.1 for all unadjusted comparisons) (Table 1). We observed a significantly lower risk of colorectal cancer among statin users than among non-users, after adjusting for age, sex, and NSAID use (Relative risk 0.86; 95% CI, 0.74 – 0.99).

We evaluated the influence of statin use on the risk of colorectal cancer according to expression of SMAD4 in the tumor (Table 2). The benefit of statin use appeared to be confined to cancers with normal SMAD4 expression (multivariate relative risk, 0.66; 95% CI, 0.53 to 0.81). In contrast, statin use did not appear to be associated with the risk of developing colorectal cancer with weak or absent SMAD4 expression (multivariate relative risk, 1.02; 95% CI, 0.87 to 1.20). 33% (103/309) of the tumors from the Statin users had normal SMAD4 expression (SMAD4–high), whereas 42% (116/274) of controls had normal SMAD4 expression. A test for heterogeneity for the association of statin use with SMAD4–high or SMAD4–low tumors found a statistically significant association (P for heterogeneity < 0.04).

The same sample of tumors was analyzed for *K-ras* and *BRAF* mutations. Of the 622 tumors analyzed, 88 showed a *BRAF* mutation and 144 a *K-ras* mutation. We evaluated the influence of statin use on the risk of colorectal cancer according to *K-ras* and *BRAF* mutation status (Table 2). There was no association between statin use and the development of cancers with mutations in *K-ras* or *BRAF* separately and no association between statin use and the presence of either mutation versus neither mutation. 77 (23%) of the tumors that developed in statin users had *K-ras* mutations and 70 (23%) of the tumors that developed in the controls (multivariate relative risk, 0.99; 95% CI, 0.91 to 1.09). 48 (14%) of the tumors that developed in statin users had *BRAF* mutations and 40 (13%) of the tumors that developed in the controls (multivariate relative risk, 0.98; 95% CI, 0.92 to 1.05). 125 (38%) of the tumors that developed in statin users had either *K-ras* mutations or *BRAF* mutations and 110 (37%) of the tumors that developed in the controls (multivariate relative risk, 0.97; 95% CI, 0.86 to 1.10).

Based on the overall incidence of colorectal cancer in these cohorts and the prevalence of SMAD4–high and SMAD4–low tumors among participants in whom we assessed SMAD4 expression, we estimated the age-standardized incidence rate for SMAD4–high and SMAD4–low colorectal cancers in relation to statin use in the entire cohort. The age-standardized incidence rate of SMAD4–high tumors was 43 per 100,000 person-years for statin users as compared with 63 per 100,000 person-years for nonusers. In contrast, the age-standardized incidence rate of SMAD4–low tumors was 88 per 100,000 person- years for statin users as compared with 84 per 100,000 person-years for nonusers.

Table 1. Baseline Characteristics of the Study Cohort

Characteristics	Statin users (N=68,948)	Controls (N=94,272)	Total (N=163,220)	p-value
Gender (%) (no.)				
Female	43 (29,666)	63 (59,357)	55 (89,023)	
Male	57 (39,282)	37 (34,915)	45 (74,197)	0.00
Median age (yr)	64 (8 – 96)	63 (0 – 102)	63	
No CRC	64 (8 – 96)	63 (0 – 102)	63	ns
CRC	69 (39 – 91)	71 (33 – 92)	70	ns
Follow-up (mean)(yr)	4.25 (293,129	4.39 (413,870)	4.3 (706,999)	ns
NSAIDs (%)(no.) ¥	37 (25,126)	37 (35,164)	60,490	ns
Hormone Replacement Therapy (%)(no.) ¶	7.6 (4,907)	9.8 (9,249)	8,6 (14,156)	ns
Inflammatory Bowel Disease (%) ╪	1.7 (1202)	1.8 (1,732)	1,8 (2,934)	ns
Diabetes Mellitus (%)(no.) ‡	27.8 (19,289)	10.4 (9,897)	17,8 (29,186)	0.00
Previous polypectomy (%)(no.) §	1.3 (885)	1.0 (928)	1.1 (1,813)	ns

¥ NSAID use was defined yes when a patient had received at least 2 prescriptions of any NSAID and/or more than 1 prescription per year of follow-up

¶ Hormone Replacement Therapy à defined by drug records via Pharmo (estrogens with or without progestogens)

╪ IBD: positive when patients use Mesalazine

‡ DM: positive when patients use insulin and/or non-insulin medication

§ Previous polypectomy: pathology diagnosis of benign neoplasia of the colon, rectum or anus (ICD-9 codes 211.3 and 211.4)

Table 2. Characteristics of the nested Study Cohort

Characteristics	Statin users (N=68,948)	Controls (N=94,272)	Total (N=163,220)	p-value	RR (95% CI)
CRC incidence					
No CRC	68,315	93,663	162,225		
Male (%) (no.)	57 (38,846)	37 (34,467)	73,313	0.03	1.00 (1.00 – 1.00)
CRC	386	609	995		
Male (%) (no.)	71 (276)	50 (306)	582	0.00	1.42 (1.28 – 1.57)
CRC for SMAD4 analysis*	309	274	583		
Male (%) (no.)	68 (211)	54 (149)	61 (360)	0.00	1.29 (1.12 – 1.48)
Cancers evaluated (%) (no.)					
KRAS mutants †	23 (77)	23 (70)	23 (144)	0.98	0.99 (0.91 – 1.09)
BRAF mutants ‡	14 (48)	13 (40)	14 (88)	0.64	0.98 (0.92 – 1.05)
KRAS/BRAF-pathway defect	38 (125)	37 (110)	37	0.71	0.97 (0.86 – 1.10)
Cancer location (%) (no.)					
Right-sided ∫	31 (86)	30 (75)	31 (161)	0.85	0.99 (0.88 – 1.11)

* some paraffin embedded tissue samples were of insufficient quality or had to little epithelial tissue to assess representative SMAD4 analysis.

† KRAS mutations were detected using melt curve analysis and sequencing

‡ BRAF mutations were analyzed with TaqMan PCR

∫ Tumor location was defined by SNOMED codes and pathology reports when available (83% of CRC)

DISCUSSION

In this large population-based cohort study we find that statin users have a small but significant reduction in the relative risk of colorectal cancer of 13%, as compared with non-users. This is consistent with the approximately 10% reduction in colorectal cancer risk seen in meta-analyses of case-control and cohort studies of the influence of statin use on colorectal cancer incidence[3]. This reduction in risk is entirely due to a reduction in the number of SMAD4–high colorectal cancers among statin users and is consistent with our previous findings from studies *in vitro* and in rodents. In contrast, there is no significant difference in the incidence of SMAD4–low colorectal cancers between users and nonusers of statins. From our previous *in vitro* and animal studies we had expected to find an increased risk of SMAD4-low colorectal cancers. However, this study does not provide supportive evidence for any significant negative effects of statins in the SMAD4-low tumor subgroup.

We found no association between the risk of colorectal cancer harboring *K-ras* or *BRAF* mutations and statin use. The efficacy of other therapies targeting the RAS/RAF pathway such as Cetuximab and Panutinumab, is dependent on the mutational status of *K-ras* and *BRAF*. While there are several other elements of this pathway that could also be assessed we have limited our analysis to those established as predicting response to the biological therapies known to target this pathway.

The association with a reduction in cancer risk is found during statin use rather than years later as found with Aspirin. This suggests an effect of statins on the later stages of colorectal adenoma or cancer. SMAD4 is lost at a late stage during the stepwise sequence

Table 3. Relative Risk of Colorectal Cancer in Relation to SMAD4 Expression.

Variable	Statin users	Controls
All colorectal cancer*		
No. of cases	309	274
Multivariate relative risk (95% CI)‡	0.86 (0.76 – 0.98)	1.0
SMAD4-positive cancer∫		
No. of cases (%)	103 (33)	116 (42)
relative risk (95% CI)	0.66 (0.53 – 0.81)	1.0
SMAD4-negative cancer§		
No. of cases (%)	206 (67)	158 (58)
relative risk (95% CI)	1.02 (0.86 – 1.19)	1.0

* all CRC samples with enough tissue representative for SMAD4 expression analysis are included

‡ Multivariate relative risks are adjusted for age, sex and calendar time

∫ Cancers with Immunohistochemical SMAD4 staining of normal intensity are classified as SMAD4-positive cancers

§ Cancers with no Immunohistochemical SMAD4 staining or with staining of low intensity are classified as SMAD4-negative cancers

from adenoma to carcinoma[19]. Our data suggest that the anticancer benefit of statins is mediated, at least in part, through effects on SMAD4 dependent signaling. SMAD4 is the central signaling element in the TGFβ, Activin and BMP pathways. *In vitro* studies where the BMP-specific inhibitor Noggin fully antagonized the effects of statins on colorectal cancer cells, point towards the BMP pathway being the essential SMAD4 dependent pathway through which statins exert their effects. Mutations in SMAD4 lead to low protein expression and are associated with a poor prognosis. Experimental studies have shown that statins, especially at high doses, have a range of other potentially antineoplastic actions; these results indicate that further work is needed to elucidate the effects of these agents and the BMP pathway (or other SMAD4 dependent pathways) on the development of colorectal cancer. The BMP pathway may regulate apoptosis[20], angiogenesis[21], cancer stem cells[22] or tumor-cell invasiveness[23]. Our study has several strengths. First, because the prescription data is collected directly from the pharmacy, errors in recall inherent to the assessment of drug use by questionnaire are avoided. Second, by performing the analysis in beta-blocker users we control for the higher rates of colorectal cancer seen in those with cardiovascular disease[18] for which most patients take statins. Failure to do this will tend to underestimate any effect of statins. Third, we have been able to corroborate our SMAD4 staining analysis methodology by comparison with SMAD4 allele loss in a large independent panel of tumors and the proportion of cancers with low SMAD4 expression is similar to those found by other investigators[19]. There are several limitations to our study. The study was observational, and makes use of anonymous patient information precluding adjustment for factors not registered in the databases such as dietary factors, smoking and body mass index. Likewise, we cannot adjust for the use of over the counter medication.

Table 4. Relative Risk of Colorectal Cancer in Relation to Statin dosage*

Statin	**Dose of Statin (mg)**				
	0	**10**	**20**	**40**	**p-value‡**
Simvastatin					
No. cancers/statin-users (%)	0.65	0.27	0.46	0.64	
relative risk (95% CI)	1.0	0.41 (0.20 – 0.87)	0.71 (0.57 – 0.88)	0.98 (0.75 – 1.29)	0.01
Atorvastatin					
No. cancers/statin-users (%)	0.65	0.47	0.50	0.40	
relative risk (95% CI)	1.0	0.73 (0.54 – 0.99)	0.77 (0.57 – 1.04)	0.63 (0.44 – 0.88)	0.51
Rosuvastatin					
No. cancers/statin-users (%)	0.65	0.15	0.15	0.21	
relative risk (95% CI)	1.0	0.24 (0.18 – 0.32)	0.23 (0.11 – 0.49)	0.32 (0.08 – 1.28)	0.80

* Dose is subdivided in the average amount of Statins taken daily by users during the study period
‡ p values are for linear trend

Our results support the importance of continued investigation into SMAD4 and related pathways for the development of new treatments and the potential use of SMAD4 as a molecular marker for tailoring therapy in patients with a history of colorectal cancer.

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