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Combination Therapy with Atorvastatin and Celecoxib Delays Tumor Formation in a Fanconi Anemia Mouse Model

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Abstract

Background—Fanconi anemia is an inherited bone marrow failure disorder associated with a high incidence of leukemia and solid tumors. Currently, no interventions to prevent or delay the formation of solid tumors are available.

Procedure—Two of the most important hallmarks of Fanconi anemia are inflammation and oxidative stress. In this study, we administered the antioxidant atorvastatin and the anti-inflammatory drug celecoxib to cohorts of *Fancd2*^{-/-}/*Trp53*^{+/-} mice, a model of Fanconi anemia. Treatment started at weaning and continued until the mice developed a palpable mass or suffered from >20% weight loss. Tumors samples and selected tissues were subjected to histopathological examination. X² test was performed to analyze tumor incidence and Kaplan-Meier survival curves were evaluated with log-rank test. In addition, a small cohort of mice was monitored for the safety of the drugs.

Results—The combined oral administration of both drugs significantly delayed tumor onset in *Fancd2*^{-/-}/*Trp53*^{+/-} mice. Specifically, the treatment delayed the onset of ovarian tumors in *Fancd2*^{-/-}/*Trp53*^{+/-} mice and increased the mean ovarian tumor-free survival time by 17%, whereas this combinatorial drug regimen did not have a significant effect on other tumor types. In addition, no detrimental effects on hematopoiesis from the drug treatment were observed during a 12-month safety monitoring.

Conclusions—The data presented here suggest that a combination of atorvastatin and celecoxib may be a good candidate for chemoprevention in Fanconi anemia.

Keywords

Tumor prevention; Fanconi anemia; Ovarian cancer

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Introduction

Fanconi anemia (FA) is a blood disorder associated with a very high incidence of leukemia and other solid tumors. FA is genetically heterogeneous, with at least 22 complementation groups and genes identified so far [1]. This complicated gene network, termed the FA network, repairs DNA lesions such as interstrand cross-links and maintains genome stability [1]. Despite the rapid progress in understanding the molecular mechanisms involved in the FA pathway in recent years, therapeutic interventions for FA patients are very limited. In particular, no interventions to prevent or delay the formation of solid tumors are currently available.

Two characteristic FA phenotypes are elevated oxidative stress and altered cytokine responses [2–4]. The up-regulation of oxidative stress in FA was first noted several decades ago and has been believed to be a critical factor contributing to the pathogenesis of bone marrow failure and tumor progression in FA [3,5]. Indeed, oral administration of antioxidants such as tempol delays tumor onset in FA mice [6]. A recent study involving a small cohort of FA children showed that antioxidant administration reduces DNA damage [7]. FA-deficient cells also express abnormally high levels of several pro-inflammatory cytokines, such as tumor necrosis factor alpha and interferon gamma, that may contribute to disease progression in FA [2,4,8,9]. In light of these observations, we hypothesized that a combination of an anti-inflammatory drug and an antioxidant might benefit FA patients and prevent tumor onset.

Atorvastatin is a cholesterol-lowering drug and also an antioxidant [10,11]. Celecoxib is a second generation of nonsteroidal anti-inflammatory drug (NSAID) used for the treatment of inflammation [12]. It is a selective inhibitor of COX-2, an important mediator of inflammation, and has an improved gastric safety profile compared to traditional (first generation) NSAIDs. Atorvastatin and celecoxib in combination have been previously shown to prevent the formation of colon, prostate, and intestinal tumors [13–15]. There is also evidence that the combination of the two drugs has a synergistic effect on tumor prevention [13,16]. Here, we tested combined dietary administration of atorvastatin and celecoxib for tumor prevention using the *Fancd2*^{-/-} mouse model, which recapitulates most of the FA patient phenotypes, including cancer susceptibility and bone marrow defects [17–19].

Materials and methods

Mice

Fancd2 and *Trp53* mutant mice were maintained on the 129S4 strain background as described before [20]. *Fancd2* and *Trp53* mutant breeding pairs were crossed to generate female *Fancd2*^{-/-}/*Trp53*^{+/-} mice and littermate *Fancd2*^{+/+}/*Trp53*^{+/-} controls. Atorvastatin and celecoxib were obtained from the Oregon Health & Science University pharmacy and added to the common rodent chow 5015 (19% protein and 11% fat) at 100 parts per million (ppm) and 300 ppm in the diet, respectively (Bio-Serv, Flemington, NJ, USA). Treatment started at weaning and continued until the mice developed a palpable mass or suffered from

>20% weight loss. All animals were treated in accordance with the guidelines of the Institutional Animals Care and Use Committee of Oregon Health & Science University.

Histopathology

Tumors samples and selected tissues (liver, spleen, thymus, ovary, and uterus) were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin as described previously [20].

Flow cytometry

Bone marrow cells were isolated from the femora and tibiae and immune-stained for the analysis of CD34⁺KSL cells as described previously [21].

Statistical analysis

For tumor suppression analysis, χ^2 test was performed. In all the other cases, P values were calculated by two-tailed, unpaired student's t -test. Both χ^2 test and t -test were done with the STATA 15.0 software (StataCorp, College Station, TX, USA). Kaplan-Meier survival curves were generated using Prism 7 software (GraphPad Software Inc, San Diego, CA, USA) and evaluated with log-rank test. A P value less than 0.05 was considered significant.

Results

We have previously shown that FA mutated *Fancd2*^{-/-}/*Trp53*^{+/-} mice have a significant increase in the incidence of epithelial tumors compared to *Fancd2*^{+/+}/*Trp53*^{+/-} non-FA controls [21,22]. The tumor incidence is higher in female than male mice of this genotype. Therefore, cohorts (30 mice per group) of female *Fancd2*^{-/-}/*Trp53*^{+/-} mice and *Fancd2*^{+/+}/*Trp53*^{+/-} controls were treated with either atorvastatin/celecoxib diet or placebo. The doses of atorvastatin and celecoxib were kept low at 100 ppm and 300 ppm respectively, and were chosen based on a previous study that noticed a chemopreventive effect from the combination of these two drugs at these doses [23]. The drugs were well tolerated in this long-term study and the animals did not show any change in weight, overall health or behavior on drug treatment. As shown in Figure 1, the atorvastatin/celecoxib treatment significantly increased the mean tumor-free survival time of *Fancd2*^{-/-}/*Trp53*^{+/-} mice by 20% ($P<0.03$), from 350 days to 420 days. *Trp53*^{+/-} without a *Fancd2* mutation are also tumor prone and develop mostly sarcomas and lymphomas [24]. However, the mean tumor-free survival time for the *Fancd2*^{+/+}/*Trp53*^{+/-} mice was not significantly different between the treatment and control groups, indicating that the tumor-delaying effect from atorvastatin/celecoxib was specific to only FA mice.

Similar to what we had seen in this mouse model previously [6,17,21], the dominant tumor type in untreated *Fancd2*^{-/-}/*Trp53*^{+/-} mice was ovarian carcinomas. In contrast, ovarian tumors were less common in *Fancd2*^{+/+}/*Trp53*^{+/-} mice. Therefore, we also analyzed the Kaplan-Meier survival curves of ovarian cancer-bearing mice. As shown in Figure 2, atorvastatin/celecoxib administration significantly delayed the onset of ovarian tumor in *Fancd2*^{-/-}/*Trp53*^{+/-} mice and increased the mean ovarian tumor-free survival time by 17% ($P=0.02$) from 397 days to 463 days. Similar to the overall tumor-free survival curves, this

tumor-delaying effect is specific to only FA mice, since the ovarian cancer survival curves for *Fancd2*^{+/+}/*Trp53*^{+/-} mice showed no significant difference between atorvastatin/celecoxib treatment and placebo treatment (Figure 2). Moreover, the tumor-delaying effect of the drug treatment on non-ovarian cancers (mainly uterine adenocarcinomas, lymphomas, osteosarcomas, and angiosarcomas) was not statistically significant ($P=0.06$; Supplemental Figure S1), possibly due to a milder effect or a smaller sample size. We then compared the ovarian cancer incidence between the drug treatment and placebo groups. The risk of having ovarian cancer for the atorvastatin/celecoxib-treated *Fancd2*^{-/-}/*Trp53*^{+/-} mice was 55.2%, whereas the risk for the placebo-treated *Fancd2*^{-/-}/*Trp53*^{+/-} controls was 67.7%. Although it appeared that the odds of having ovarian cancer in atorvastatin/celecoxib group was lower than the odds in placebo group (odds ratio: 0.59; 95% confidence interval: 0.21 – 1.69; $P=0.32$), the results were not statistically significant, indicating that atorvastatin/celecoxib didn't significantly suppress the ovarian tumor formation in *Fancd2*^{-/-}/*Trp53*^{+/-} mice.

In addition to the cancer prevention study described above, these drugs were also evaluated for their effects on the hematological phenotypes of *Fancd2*^{-/-} mice in a cohort of ten mice that did not carry any *Trp53* mutation. Animals were started with atorvastatin/celecoxib diet at weaning and kept on the diet for 12 months. At harvest, bone marrow cells were analyzed by flow cytometry to measure the size of the hematopoietic stem cell compartment. As shown in Figure 3, the *Fancd2*^{-/-} mice treated with either atorvastatin/celecoxib diet or placebo diet showed the same number of CD34⁺KSL cells, the immunophenotypically-defined hematopoietic stem cells measured by flow cytometry, indicating no beneficial or detrimental effects on hematopoiesis from the drug treatment.

Discussion

Two of the most important hallmarks of FA phenotypes are inflammation and oxidative stress [2–5,7–9]. Here we tested a combination therapy of antioxidant atorvastatin and anti-inflammatory drug celecoxib in a mouse model of Fanconi anemia. This therapy significantly delayed the tumor onset in *Fancd2*^{-/-}/*Trp53*^{+/-} mice. Consistent with previous observations that disruption of FA pathway was associated with elevated ovarian cancer risk [25–27], most of the cancers developed by *Fancd2*^{-/-}/*Trp53*^{+/-} mice were ovarian in origin, whereas ovarian cancers were rare in wildtype mice. Impressively, the dietary atorvastatin/celecoxib administration significantly delayed the onset of ovarian tumors in *Fancd2*^{-/-}/*Trp53*^{+/-} mice and increased the mean ovarian tumor-free survival time by 17%. This is not surprising, considering that inflammation might be involved in the initiation, promotion and progression of ovarian cancers [28,29]. In addition, a recent study has suggested that atorvastatin might have anti-tumorigenic effect on ovarian cancer cells *in vitro* [30]. Further investigation, especially a test of either atorvastatin or celecoxib alone, is warranted to fully understand the mechanism behind this drug combination's tumor-delaying effect.

As a cholesterol-lowering drug for more than three decades, atorvastatin has a good safety profile and is generally well tolerated [31], although a higher risk of liver enzyme elevation (without clinically important adverse consequences) is associated with a long-term use of atorvastatin at 80 mg/day [32]. The side effects of the NSAIDs generally range from moderate GI toxicity to cardiotoxicity. As a second-generation NSAID, celecoxib has an

improved safety profile and is the only NSAID with FDA-approval for patients with familial adenomatous polyposis [33,34]. In our current study, atorvastatin and celecoxib were used in combination at a much lower dose than the commonly used dose for either drug when administered individually (atorvastatin: 150 to 200 ppm; celecoxib: often 500 to 600 ppm, up to 1500 ppm) [23,35,36]. This strategy has been proven to be more effective in suppressing (colon and prostate) cancers than higher doses of either drug alone and also minimize the potential toxicity or adverse side effects [15,37]. Indeed, under our experimental conditions, no detrimental effects on hematopoiesis from the combinatorial drug treatment were observed during a 12-month safety monitoring. It is also worth noting that, on the basis of the body surface area conversion, the atorvastatin and celecoxib doses used in this animal study correspond to 0.67 mg/kg/day for atorvastatin and 2 mg/kg/day for celecoxib in humans, well below the standard doses (10–80 mg/day for atorvastatin and 200–400 mg/day for celecoxib) recommended for young children.

The development of head and neck squamous cell carcinoma is also closely associated with inflammation [38,39]. Celecoxib might be a promising chemopreventive agent for head and neck squamous cell carcinoma [39]. However, head and neck cancers are rarely seen in our FA mouse model. It therefore remains to be determined whether atorvastatin/celecoxib administration can suppress or delay the onset of head and neck squamous cell carcinomas.

Collectively, the data presented here suggest that atorvastatin and celecoxib in combination might be a good candidate for chemoprevention in FA. It is also worth considering the combination of these compounds with other promising drug candidates for FA such as metformin, which both improves hematopoiesis and delays tumor onset [21], since the combination of atorvastatin and metformin has recently been shown to synergistically delay tumors [40].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations used:

FA	Fanconi anemia
NSAID	nonsteroidal anti-inflammatory drug

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Figure 1: Atorvastatin/celecoxib administration delayed tumor onset in *Fancd2*^{-/-}*Trp53*^{+/-} mice. A). Kaplan-Meier survival curves of the *Fancd2*^{-/-}*Trp53*^{+/-} mice. The data represent 31 mice for placebo treatment and 29 mice for atorvastatin/celecoxib treatment. B). Kaplan-Meier survival curves of the *Fancd2*^{+/+}*Trp53*^{+/-} mice. The data represent 30 mice for placebo treatment and 30 mice for atorvastatin/celecoxib treatment. Tumor and selected tissues were fixed in 10% phosphate-buffered formalin and stained with hematoxylin and eosin before being examined under a microscope. The Kaplan-Meier survival curves were generated by Prism 7.0 Software and *P* values were calculated using the Log-rank (Mantel-Cox) test. NS, ATO, and CEL denote not significant, atorvastatin, and celecoxib, respectively.

A Ovarian tumor-free survival of *Fancd2*^{-/-}*p53*^{+/-} mice

B Ovarian tumor-free survival of *Fancd2*^{+/-}*p53*^{+/-} mice

Figure 2: Atorvastatin/celecoxib administration delayed the onset of ovarian tumors in *Fancd2*^{-/-}*Trp53*^{+/-} mice.

A). Kaplan-Meier ovarian tumor-free survival curves of the *Fancd2*^{-/-}*Trp53*^{+/-} mice. The data represent 31 mice for placebo treatment and 29 mice for atorvastatin/celecoxib treatment. B). Kaplan-Meier ovarian tumor-free survival curves of the *Fancd2*^{+/-}*Trp53*^{+/-} mice. The data represent 30 mice for placebo treatment and 30 mice for atorvastatin/celecoxib treatment. NS denotes not significant. NS, ATO, and CEL denote not significant, atorvastatin, and celecoxib, respectively.

Figure 3: Atorvastatin/celecoxib administration generated no beneficial or detrimental effects on hematopoiesis in *Fand2*^{-/-} mice.

A). Representative flow cytometric profiles of CD34⁺KSL cell frequencies in bone marrow after 12-months of atorvastatin/celecoxib administration. The cells were gated on PI⁺Lin⁻ first. B). Quantification of the CD34⁺KSL cell frequencies. Data represent 5 mice for each group. PI, NS, ATO, and CEL denote propidium iodide, not significant, atorvastatin, and celecoxib, respectively.