



## SHORT COMMUNICATION

## LRAT Promoter Hypermethylation as a Prognostic Marker for Colorectal Cancer Impairs Retinol Metabolism

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### Abstract

Retinoids and its derivatives are known to regulate tumor progression. Our previous study in Colorectal Cancer (CRC) has shown that the expression of *LRAT*, a gene converts excess retinol into retinyl ester to balance retinoids homeostasis, may be regulated by its promoter methylation status to modulate the retinoids synthesis. In this report, we begin to explore the potential mechanism of *LRAT* mediated retinoid metabolism. Our data indicate CRC patient of *LRAT* hypermethylation associated with better prognosis. A consistent finding is shown in siRNA mediated *LRAT* silencing, which leads to slow growth of CRC cell lines. We have also observed favorable CRC prognosis occurred in patients of both *LRAT* and *RAR*-beta hypermethylation, suggesting the better CRC prognosis may be mediated through *RAR beta* independent pathway.

### Keywords

CpG methylation, Thermostable ligase, Colorectal cancer, *LRAT*

### Abbreviations

*LRAT*: Lecithin Retinol Acyltransferase; *RAR*: Retinoic Acid Receptor

### Introduction

The retinoids, a group of compounds that include retinol (vitamin A) and its metabolites, play a fundamental role in normal cell growth, differentiation and apoptosis [1-4]. Retinol is regularly obtained from the diet. In animal products such as meat and eggs, retinol is stored in the form of retinyl esters. Upon digestion, the retinyl esters are released and subsequently hydro-

lyzed to retinol. Retinol can also be synthesized from the pro-vitamin A carotenoids ( $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin) found in fruits and vegetables such as carrots, cantaloupe, broccoli and dark green leafy vegetables. In the cytosol, retinol is oxidized to retinaldehyde by Retinol Hydrogenases (*RDHs*) or Alcohol Dehydrogenases (*ADHs*) and then to retinoic acid by Retinaldehyde Dehydrogenases (*RALDHs*). Excess of retinol can be converted to retinyl esters by the enzyme Lecithin:Retinol Acetyltransferases (*LRAT*) and stored in the liver.

Colorectal Cancer (CRC) is one of the leading causes of cancer related deaths. Studies have shown altering retinol metabolism may have chemotherapeutic benefits on CRC [1,5-8]. For example, the most active metabolite of retinol, retinoic acid, is transported to cell nucleus where it binds to the Retinoic Acid Receptors (*RAR*  $\alpha, \beta, \gamma$ ), stimulates downstream gene expression, affects cell cycle progression, and leads to cancer cell growth inhibition. The challenge of using retinoic acid in cancer chemotherapy is that the expression of *RARs* is often lost at advanced tumor stages, rendering the poor efficacy of retinoic acid treatment. This phenomenon of retinoic acid resistance has been shown partly due to epigenetic changes such as aberrant histone modification and DNA methylation to silence *RAR* gene expression.

Previously, we have shown frequent *LRAT* hypermethylation in earlier (I/II) than in later (III/IV) CRC stag-

es [9]. This inverse relationship between CRC stages and the hypermethylated *LRAT* instances is a tumor-specific, non-random event ( $p < 0.0001$ ). We have also demonstrated that *LRAT* hypermethylation is independent of tumor MSI status; therefore, this *LRAT* methylation pattern is not a typical MSI feature of possessing many hypermethylated genomic loci. In this study, we begin to explore the aberrant *LRAT* hypermethylation and its relationship to CRC clinical outcomes.

## Results and Discussion

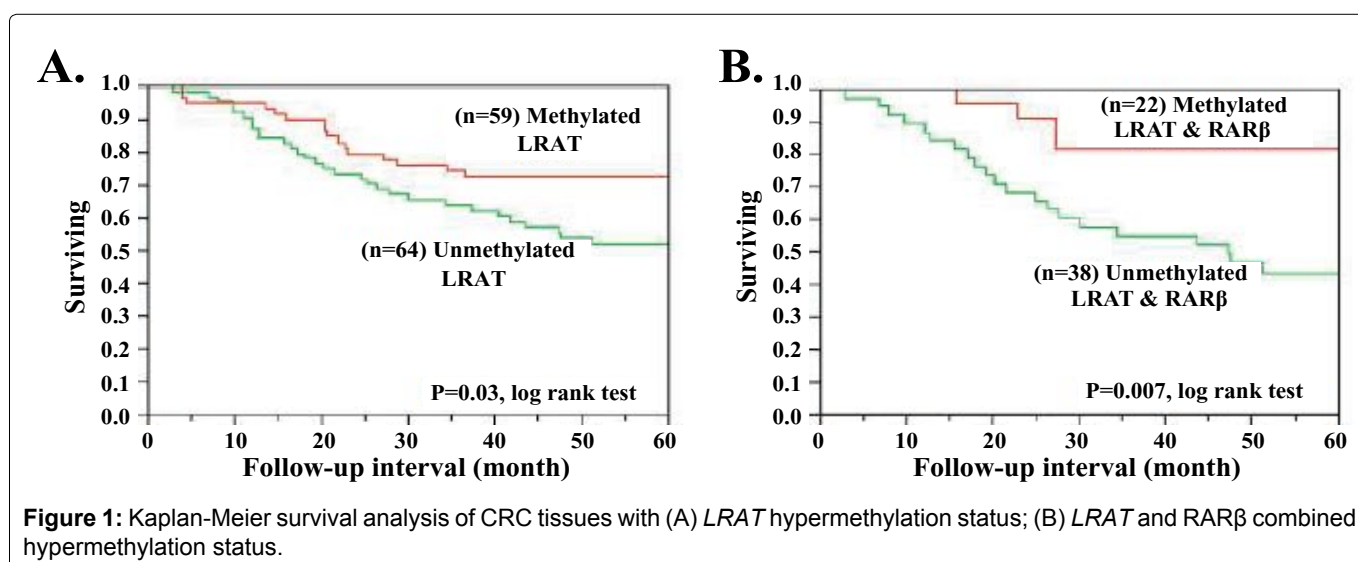
In many cancers, the majority of aberrant promoter hypermethylation are positively correlated with tumor stages [10]. Namely, the high instances of hypermethylated promoters are more often seen in late tumor stages, such as RAR promoter hypermethylation seen in CRC. Since patients with early-stage CRC are known to have better outcomes [11], and the high instances of *LRAT* hypermethylation occurs in early-stage CRC, one may predict a favorable prognosis of *LRAT* hypermethylated CRC cases. As shown in Figure 1A, Kaplan-Meier survival analysis was performed on 123 CRC cases with known *LRAT* methylation status. All these samples are microsatellite stable and none colonic polyps, ensuring the survival analysis is not biased towards a better outcome (e.g. MSI) or skewed towards early CRC stages (e.g. polyps). Interestingly, patients with *LRAT* hypermethylation did present a favorable prognosis ( $p = 0.03$ ). Consistent with our finding, a recent study has also demonstrated high expression of *LRAT* in melanoma metastases was inversely correlated with patient survival [12]. Our data suggest that the frequent *LRAT* hypermethylation may represent a useful predictor to stratify CRC. Since *LRAT* promoter hypermethylation results in decreased gene expression, we hypothesize that reduced *LRAT* gene expression may involve a defending or counteracting mechanism to CRC tumorigenesis.

To further investigate the impact of reduced *LRAT* expression on the growth of CRC cells, DLD1 and HT29 cell lines were transfected with *LRAT* small interfering

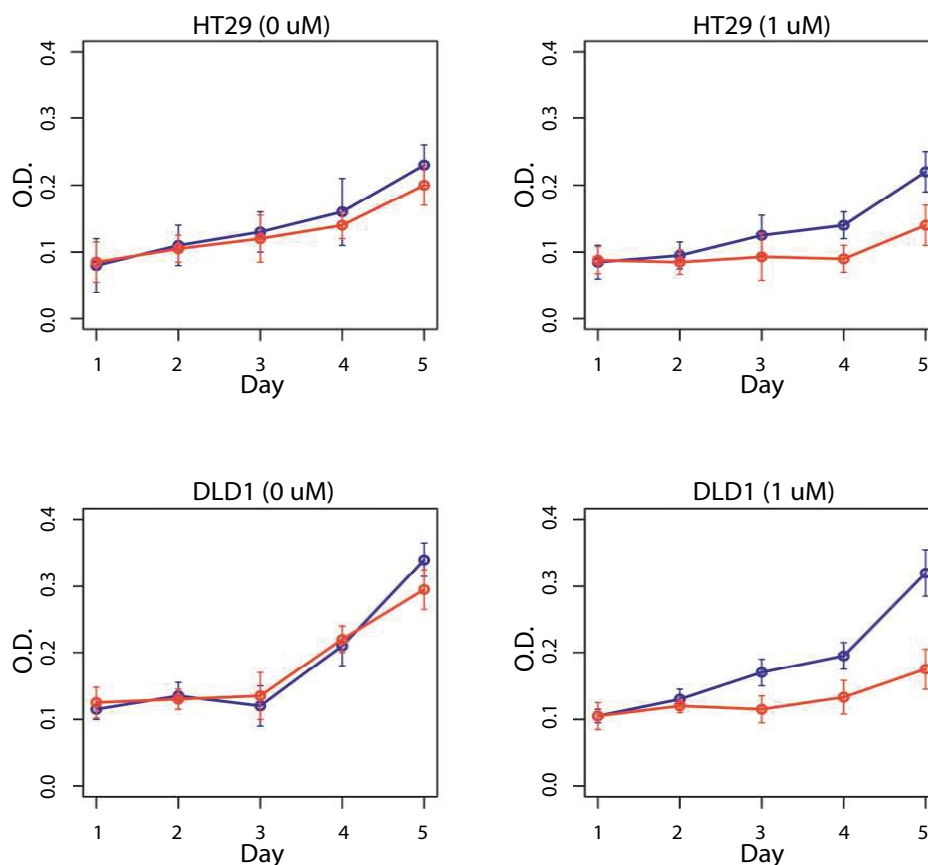
RNA (siRNA) which was designed to target the human *LRAT* sequence (NM\_004744) position 3272-3296, with the sense targeting sequence: CAAGGAGGGAGGAU-CA-CAAGGUCAG. A duplex Dicer-substrate siRNA with a scrambled sequence (DS ScrambledNeg) that does not target any sequence in the human genome was used as a universal negative control. For each condition,  $3 \times 10^3$  CRC cells were seeded in each well of the 96-well plates on day one and assayed in triplicates. A final 10 nM siRNAs were transfected into DLD1 and HT29 cells using RNAiMax (Invitrogen) under the manufacturer recommended condition, in the presence or absence of retinol. *LRAT* RNAs were measured 48 hours after transfections using qRT-PCR to determine the transfection and knockdown efficiencies. Cells were harvested every 24 hours after the first two-day of transfection and subjected to a MTT cell proliferation assay (ATCC).

As shown in Figure 2, in the absence (0  $\mu$ M) of retinol, there was no difference in cell proliferation between *LRAT* knockdown and control cases. Remarkably, in the presence of 1  $\mu$ M retinol, *LRAT* knockdown cells showed slower growths than the controls. These results imply that reduced *LRAT* expression is linked to a slow proliferation of CRC cells mediated by retinol, and may underlie the association between *LRAT* methylation silencing and a better prognosis of CRC.

An explanation to *LRAT*-involved slow cell proliferation may attribute to mechanisms of retinol mediated CRC growth inhibition. Reduced *LRAT* expression impairs the esterification of excess retinol. The unconverted retinol is then metabolized into retinoic acid and contributes to antiproliferation effects likely through a Retinoic Acid Receptor (RAR) pathway [13]. Alternatively, several publications have proposed another mechanism suggesting a RAR-independent pathway for retinol-mediated growth inhibition of CRC and melanoma cells [1,2,14]. Our data also suggest that *LRAT* methylation silencing facilitates the utilization of retinol in regulating cellular proliferation. Furthermore, our analysis of RAR methyl-



**Figure 1:** Kaplan-Meier survival analysis of CRC tissues: with (A) *LRAT* hypermethylation status; (B) *LRAT* and RAR $\beta$  combined hypermethylation status.



**Figure 2:** siRNA targets *LRAT* in CRC cell lines. The optical density was measured at 450 nm for cell proliferation assay. Red and blue curves represent the presence or absence of *LRAT* interference treatment, respectively. Retinol concentration used for each assay is shown in the parenthesis next to the cell line name.

ation status has shown that CRC patients with promoter hypermethylation in both *LRAT* and *RARβ* correlated with a better prognosis ( $p = 0.007$ , Figure 1B). Although this finding does not preclude the participation of *LRAT* in the anti-proliferation mechanism involving retinoic acid and its receptors, the association between a good survival outcome and hypermethylation at both *LRAT* and *RARβ* loci suggests that methylation silenced *LRAT* may be a key feature in a *RAR*-independent pathway to tumorigenesis inhibition.

*LRAT* activity is governed by different mechanisms. Vitamin A and its main metabolite (retinoic acid) regulate both *LRAT* mRNA and gene activity in a tissue specific manner [15]. Study in animal model suggested a second mechanism of regulating *LRAT* expression via CRBP. The ratio of free CRBP to holo-CRBP directly reflects vitamin A concentration and influence its regulatory ability [16,17]. Here, we propose *LRAT* promoter hypermethylation as another mechanism for modulating its gene expression. It has been proposed that *LRAT*, in conjunction with cytochrome oxidases P450s, plays an essential role in regulating the availability of retinol as a precursor for retinoic acid [16]. Methylation silencing of *LRAT* may abolish the feedback regulation and disrupt the homeostasis of a careful balance between retinoic acid and retinol concentration. Consequently, such an imbalance may interfere with regular cell growth, dif-

ferentiation and apoptosis. Future studies to investigate *LRAT*'s roles in each of the retinol mediated inhibitory mechanisms will shed light on this interesting topic.

## Acknowledgements

Funding for this work was provided by the Clinical Nutrition Research Unit P30-CA29502 (YWC), National Cancer Institute P01-CA65930 (FB), Ludwig Institute for Cancer Research/Conrad N. Hilton Foundation joint Hilton-Ludwig Cancer Metastasis Initiative, and the Gilbert Family Foundation. The authors thank WeiJi Shi for the advice in statistical analysis. We also thank Owen Parker and Jianmin Huang for insightful discussion and critical reading of the manuscript.

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