Nutraceuticals Mediated Regulation of microRNAs for Cancer Therapy

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There is no cure for metastatic cancer

Cancer cells that leads to metastasis are highly heterogeneous

Among these heterogeneous cells, EMT phenotypic cells, Cancer Stem Cells (CSCs) or Cancer Stem-Like Cells (CSLCs) are becoming novel targets

EMT, CSCs or CSLCs are highly resistant to conventional therapies, and these are the cells that leads to tumor recurrence and metastasis

Therefore, strategies (nutraceuticals? - Naturopathy) for targeted elimination of these resistant cells would lead to eradicate tumors and metastatic disease
Most therapies fail to consider the difference in drug sensitivities of cancer stem cells compared to their non-tumorigenic progeny.

Most therapies target rapidly proliferating non-tumorigenic cells and spare the relatively quiescent cancer stem cells.
Tumor Microenvironment

HIFs

Cancer Stemness Markers (Oct4, Nanog, Sox2 and Notch)

EMT Markers (ZEB2, Snail, Twist, Wnt, Slug, Notch & TGF-β)

Epithelial Cells
Mesenchymal Cells
Cancer Stem Cells

Tumor Aggressiveness
Tumor growth, Invasion, Metastasis, Therapy Resistance and Recurrence
Different Compartment of Cancer Cells

Intrinsic

- CSC with metastatic potential

Induced

- EMT
- Transition to CSC-like state
- Recruitment of reactive stroma
- Cells poised to undergo EMT

Chaffer and Weinberg, Science, 2011
Biogenesis of microRNAs (miRNAs)

1. **Transcription by RNA Pol II or III**: Transcription of the miRNA gene
2. **Cleavage by Drosha**: The pri-miRNA is cleaved by Drosha to form a pre-miRNA.
3. **Export to cytoplasm**: The pre-miRNA is exported from the nucleus to the cytoplasm through the Exportin 5 pathway.
4. **Cleavage by Dicer**: The pre-miRNA is further cleaved by Dicer to form the miRNA duplex.
5. **Degradation of mRNA**: The mature miRNA can degrade target mRNAs.
6. **Inhibition of translation**: The mature miRNA can inhibit the translation of target mRNAs.

**Key Players**:
- RNA Pol II or III
- Drosha
- DGCR8
- Exportin 5
- Dicer
- TRBP
- Ago2
- RISC
Loss of miR-200, Let-7 and miR-34 family are highly prevalent in aggressive tumors, and thus up-regulation or re-expression of these miRNAs by natural agents (nutraceuticals) would become a novel strategy for eradicating tumors, which will prevent tumor recurrence, and this will improve overall survival.
Isoflavones & Indoles
Isoflavones (majority comes from soybean)

A. Glycosides (in plants) → Aglycones
   Ingestion
   glucosidases

B. 17β-estradiol

Genistein
Daidzein
and other aglycones
Indole and its *in vivo* metabolite (BR-DIM)
Premise:

Loss of miR-200, Let-7 and miR-34 family are highly prevalent in aggressive tumors, and thus up-regulation or re-expression of these miRNAs by natural agents (nutraceuticals) would become a novel strategy for eradicating tumors, which will prevent tumor recurrence, and thus will improve overall survival.
Let-7 regulates EZH2 expression by binding to EZH2 3’UTR

**A**

Let-7 binding sites in 3’UTR of EZH2 mRNA

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>3’UTR Sequence</th>
<th>miRNA</th>
<th>EZH2</th>
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<tr>
<td>3’</td>
<td>uugauauguUGGAUGAUGAUGAGu 5’</td>
<td>hsa-let-7a</td>
<td></td>
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<tr>
<td>1:5’</td>
<td>cAUCUGCUACCUCc</td>
<td></td>
<td>EZH2</td>
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<td>EZH2</td>
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</table>

**B**

EZH2 3’UTR luciferase activity (% of con)

![EZH2 3’UTR luciferase activity graph](image)

**C**

**PC3 cells**

<table>
<thead>
<tr>
<th>Group</th>
<th>Con</th>
<th>let-7a</th>
<th>let-7b</th>
<th>Pre-miRNA</th>
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<td></td>
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<td>GAPDH</td>
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**PC3 PDGF-D cells**

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Re-expression of miRNAs in prostate cancer patients by BR-DIM intervention

A
Relative let-7a levels (log2)
Patient  Patient + BR-DIM
p = 0.0875

B
Relative let-7b levels (log2)
Patient  Patient + BR-DIM
p = 0.026

C
Relative let-7c levels (log2)
Patient  Patient + BR-DIM
p = 0.0001

D
Relative let-7d levels (log2)
Patients  Patients + BR-DIM
p = 0.0337

Relative levels of EZH2 mRNA (log2)
Patients  Patients + BR-DIM
p = 0.0404
Re-expression of miRNAs in prostate cancer patients by BR-DIM intervention

A. Relative levels of miR-34a

B. Relative levels of AR mRNA

C. Relative levels of PSA mRNA

D. Relative levels of Notch1 mRNA

Legend:
- Patients
- Patients+BR-DIM

P-values:
- miR-34a: p=0.0398
- AR mRNA: p=0.0154
- PSA mRNA: p=0.1973
- Notch1 mRNA: p=0.0114
Down-regulation of AR and its nuclear exclusion by BR-DIM intervention in prostate cancer patients
How the expression of miRNAs are regulated?
Epigenetic regulation of miRNAs

A

<table>
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<tr>
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<th>C4-2B</th>
<th>LNCaP</th>
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<tr>
<td>Control</td>
<td><img src="image1" alt="Graph" /></td>
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<tr>
<td>5-aza-dC</td>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
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B

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<tbody>
<tr>
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<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
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<tr>
<td>BR-DIM</td>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
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C4-2B and LNCaP cell lines were treated with 5-aza-dC or BR-DIM, and the relative levels of miR-34a were measured.
Isoflavone reduces methylation of miR-29a and miR-1256 promoter and increases expression of these miRNAs

C: control; A: Aza-dC; Iso: isoflavone; M: methylation; U: unmethylation
B-DIM and isoflavone up-regulates let-7b and let-7e expression in pancreatic cancer cells

Abstract

The emergence of castrate-resistant prostate cancer (CRPC) contributes to the high mortality of patients diagnosed with prostate cancer (PCa), which in part could be attributed to the existence and the emergence of cancer stem cells (CSCs). Recent studies have shown that deregulated expression of microRNAs (miRNAs) contributes to the initiation and progression of PCa. Among several known miRNAs, let-7 family appears to play a key role in the recurrence and progression of PCa by regulating CSCs; however, the mechanism by which let-7 family contributes to PCa aggressiveness is unclear. Enhancer of Zeste homolog 2 (EZH2), a putative target of let-7 family, was demonstrated to control stem cell function. In this study, we found loss of let-7 family with corresponding over-expression of EZH2 in human PCa tissue specimens, especially in higher Gleason grade tumors. Overexpression of let-7 by transfection of let-7 precursors decreased EZH2 expression and repressed clonogenic ability and sphere-forming capacity of PCa cells, which was consistent with inhibition of EZH2 3'UTR luciferase activity. We also found that the treatment of PCa cells with BR-DIM (formulated DIM: 3,3'-diindolylmethane by Bio Response, Boulder, CO, abbreviated as BR-DIM) up-regulated let-7 and down-regulated EZH2 expression, consistent with inhibition of self-renewal and clonogenic capacity. Moreover, BR-DIM intervention in our on-going phase II clinical trial in patients prior to radical prostatectomy showed upregulation of let-7 consistent with down-regulation of EZH2 expression in PCa tissue specimens after BR-DIM intervention. These results suggest that the loss of let-7 mediated increased expression of EZH2 contributes to PCa aggressiveness, which could be attenuated by BR-DIM treatment, and thus BR-DIM is likely to have clinical impact.
Dejuan Kong, Elisabeth Heath, Wei Chen, Michael Cher, Isaac Powell, Lance Heilbrun, Yiwei Li, Shadan Ali, Seema Sethi, Oudai Hassan, Clara Hwang, Nilesh Gupta, Dhananjay Chitale, Wael A Sakr, Mani Menon, Fazlul H Sarkar:


Abstract:
Androgen Receptor (AR) signaling is critically important during the development and progression of prostate cancer (PCa). The AR signaling is also important in the development of castrate resistant prostate cancer (CRPC) where AR is functional even after androgen deprivation therapy (ADT); however, little is known regarding the transcriptional and functional regulation of AR in PCa. Moreover, treatment options for primary PCa for preventing the occurrence of CRPC is limited; therefore, novel strategy for direct inactivation of AR is urgently needed. In this study, we found loss of miR-34a, which targets AR, in PCa tissue specimens, especially in patients with higher Gleason grade tumors, consistent with increased expression of AR. Forced over-expression of miR-34a in PCa cell lines led to decreased expression of AR and prostate specific antigen (PSA) as well as the expression of Notch-1, another important target of miR-34a. Most importantly, BR-DIM intervention in PCa patients prior to radical prostatectomy showed re-expression of miR-34a, which was consistent with decreased expression of AR, PSA and Notch-1 in PCa tissue specimens. Moreover, BR-DIM intervention led to nuclear exclusion both in PCa cell lines and in tumor tissues. PCa cells treated with BR-DIM and 5-aza-dC resulted in the demethylation of miR-34a promoter concomitant with inhibition of AR and PSA expression in LNCaP and C4-2B cells. These results suggest, for the first time, epigenetic silencing of miR-34a in PCa, which could be reversed by BR-DIM treatment and, thus BR-DIM could be useful for the inactivation of AR in the treatment of PCa.

Abstract

The epigenetic regulation of genes has long been recognized as one of the causes of prostate cancer (PCa) development and progression. Recent studies have shown that a number of microRNAs (miRNAs) are also epigenetically regulated in different types of cancers including PCa. In this study, we found that the DNA sequence of the promoters of miR-29a and miR-1256 are partly methylated in PCa cells, which leads to their lower expression both in PCa cells and in human tumor tissues compared with normal epithelial cells and normal human prostate tissues. By real-time PCR, Western Blot analysis and miRNA mimic and 3'-UTR-Luc transfection, we found that TRIM68 is a direct target of miR-29a and miR-1256 and that the downregulation of miR-29a and miR-1256 in PCa cells leads to increased expression of TRIM68 and PGK-1 in PCa cells and in human tumor tissue specimens. Interestingly, we found that a natural agent, isoflavone, could demethylate the methylation sites in the promoter sequence of miR-29a and miR-1256, leading to the upregulation of miR-29a and miR-1256 expression. The increased levels of miR-29a and miR-1256 by isoflavone treatment resulted in decreased expression of TRIM68 and PGK-1, which is mechanistically linked with inhibition of PCa cell growth and invasion. The selective demethylation activity of isoflavone on miR-29a and miR-1256 leading to the suppression of TRIM68 and PGK-1 expression is an important biological effect of isoflavone, suggesting that isoflavone could be a useful non-toxic demethylating agent for the prevention of PCa development and progression.
Summary

Primary tumor → Chemotherapeutic reagents → Metastasis

EZH2

Let-7

BR-DIM

Chemotherapeutic reagents

Cancer stem cell

Differentiated cancer cells

Dead cells
Curcumin analogue (CDF)
Curcumin analogue

Curcumin-difluorinated (CDF)

Chemical Formula: $\text{C}_{28}\text{H}_{22}\text{F}_2\text{O}_6$
Exact Mass: 492.14
Molecular Weight: 492.47
$m/z$: 492.14 (100.0%), 493.14 (30.8%), 494.15 (4.6%), 494.14 (1.2%)
Elemental Analysis: C, 68.29; H, 4.50; F, 7.72; O, 19.49
CDF

miR-200

EMT

Akt

NF-κB

ZEB1

E-cadherin

Abstract
The histone methyltransferase EZH2 is a central epigenetic regulator of cell survival, proliferation, and cancer stem cell (CSC) function. EZH2 expression is increased in various human cancers, including highly aggressive pancreatic cancers, but the mechanisms underlying for its biologic effects are not yet well understood. In this study, we probed EZH2 function in pancreatic cancer using diflourinated-curcumin (CDF), a novel analogue of the turmeric spice component curcumin that has antioxidant properties. CDF decreased pancreatic cancer cell survival, clonogenicity, formation of pancreatospheres, invasive cell migration, and CSC function in human pancreatic cancer cells. These effects were associated with decreased expression of EZH2 and increased expression of a panel of tumor-suppressive microRNAs (miRNA), including let-7a, b, c, d, miR-26a, miR-101, miR-146a, andmiR-200b, c that are typically lost in pancreatic cancer. Mechanistic investigations revealed that reexpression of miR-101 was sufficient to limit the expression of EZH2 and the proinvasive cell surface adhesion molecule EpCAM. In an orthotopic xenograft model of human pancreatic cancer, administration of CDF inhibited tumor growth in a manner associated with reduced expression of EZH2, Notch-1, CD44, EpCAM, and Nanog and increased expression of let-7, miR-26a, and miR-101. Taken together, our results indicated that CDF inhibited pancreatic cancer tumor growth and aggressiveness by targeting an EZH2-miRNA regulatory circuit for epigenetically controlled gene expression.
Natural agents alter the expression of miRNAs that are known to regulate cellular signaling and biological behavior.
Epigenetic reprogramming by natural agents

miR-200b
miR-200c

Suz12
EED
EZH2

HDAC

BR-DIM

Let-7

Suz12
EED
EZH2

DNMT

Isoflavone

AC-H3

AC-H3

CDF

Epigenetic reprogramming by natural agents

Me
Me
Me
Me
Me
Me
Me
Me
Me
CG

off

EMT & Stemness

Tumor cell aggressiveness
State of Our Knowledge on Cancer Care

Prevention → Treatment → Prevention

- Partial Response
- Progression → Tumor reemergence and Metastasis
- Dormant → No Metastasis
- Complete Response → Tumor Cured

[This is what we must achieve]
Thank You!!!

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Ms. Sadan Ali
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Dr. Judith Abrams (Statistician)
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