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Epigenetic regulation of miRNA-Cancer Stem Cells nexus by Nutraceuticals

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Abstract

Nutraceuticals, the bioactive food components represented by many naturally occurring dietary compounds, have been investigated for a few decades for their numerous beneficial effects, including their anticancer properties. The initial interest in the cancer-preventing/therapeutic ability of these agents was based on their ability to affect multiple signaling pathways that are deregulated in cancer cells. With a shift in the focus of cancer research to the emerging areas such as epigenetic regulation, microRNAs (miRNAs) and the cancer stem cells (CSCs), nutraceuticals initially appeared out of place. However, research investigations over the last several years have slowly but firmly presented evidence that supports a relevance of these agents in modern day research. While nutraceuticals are increasingly being realized to alter miRNA/CSCs expression and function, the molecular mechanism(s) are not very clearly understood. Epigenetic regulation is one mechanism by which these agents exert their anticancer effects. In this focused mini review, we summarize our current understanding of epigenetic regulation of miRNAs and CSCs by nutraceuticals. We discuss both direct and indirect evidences that support such an activity of these compounds.

Keywords

nutraceuticals; epigenetic regulation; miRNA; cancer stem cells

Introduction

The research on the connection between epigenetics and cancer has come a long way since the first reports exactly 30 years back in the year 1983 that observed lower DNA methylation [1] and lower 5-methylcytosine levels [2] in human tumors, compared to normal tissues. In these three decades the number of publications on cancer epigenetics have increased exponentially [3]. DNA methylation is now being pursued for diagnosis, prognosis as well as prediction of response to therapies in the management of cancer patients in clinics [4]. Although the term ‘epigenetics’ was originally coined to define heritable changes in phenotype independent of changes in DNA sequence, it is now used to broadly describe

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alterations in human chromatin that influence DNA-templated processes [5]. Epigenetic changes include multiple processes that can have lasting effects on gene expression without any changes in the DNA sequence itself. The chromatin represents a complex of DNA with histone proteins around which the whole genome is packaged. Epigenetic changes involve at least four known modifications of DNA and sixteen classes of histone modifications [5, 6].

A number of factors contribute to initiation and progression of human cancers resulting in the alterations of cellular machinery so as to benefit the tumor cells. The end result of all these changes is the observed resistance to apoptosis, de-regulated cell cycle and activation of signaling pathways that promote proliferation, invasion, angiogenesis and resistance to drugs. Such wide range of altered cellular functions points towards a more fundamental change in cells' genome and this has sparked an interest in the role of epigenetic regulation in initiation as well as progression of cancer [7-9]. Cancer cells, very often, are marked by activation of oncogenes as well as suppression of tumor suppressors, all of which are now believed to be regulated by multiple epigenetic changes in the chromatin. Additionally, there is a connection between inflammation and cancer [10-12]; and inflammation itself is epigenetically regulated by multiple factors [13]. The growing importance of epigenetics in cancer research and treatment is supported by the clinical approval of several drugs that target epigenetic changes. Such drugs include those that inhibit histone deacetylases and methyltransferases. The epigenetic regulation of human cancers is being advocated for its potential exploitation as diagnostic and/or therapeutic target [4, 14-25].

A number of drugs, including synthetic and natural compounds, seem to possess ability to modulate epigenetic changes [15, 26-29]. These compounds inhibit various components of cellular machinery that are responsible for the observed epigenetic changes. While the synthetic compounds have shown some promise, the focus of this article will be on the natural compounds, the compounds obtained from nature; also called 'nutraceuticals [30, 31]' or the 'bioactive food components [32]'. The benefits of nutraceuticals in cancer research are many – they are relatively non-toxic and, being part of many natural diets, are usually well tolerated.

Nutraceuticals and epigenetics

Nutraceuticals are no new comers to cancer research. The anticancer activities of a large number of such compounds is well documented and beyond the scope of this article. Until a few years ago, a major focus of anticancer research was on the ability of these compounds to modulate multiple cellular signaling pathways. With the realization of the importance of epigenetics in determination of cellular functionality, a number of nutraceuticals are now under investigation for their ability to counter epigenetic changes that accompany the onset and progression of cancer [33-46].

Most of the nutraceuticals initially attract the attention of cancer researchers through the reports on their antioxidant potential. Interestingly, the antioxidant activity of nutraceuticals itself is now believed to involve some level of epigenetic regulation. For example, there are reports on the ability of multiple nutraceuticals to epigenetically activate antioxidant Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) via interfering with the methylation status

and histone modifications [47]. First of all, a CpG island in Nrf2's promoter region was reported to be methylated in TRAMP (transgenic adenocarcinoma of mouse prostate) mice that spontaneously develop prostate cancer [48]. With the discovery of this epigenetic change that reduced the expression of Nrf2, a question was asked - whether the mechanism of action of nutraceuticals, the potent antioxidants and anticancer agents, includes reversal of Nrf2 methylation i.e. reversal of epigenetic alterations. Indeed, a number of nutraceuticals, such as curcumin [49], sulforaphane [50], z-ligustilide [51] as well as DIM (3,3'-diindolylmethane) [52] have since been shown to epigenetically activate Nrf2 through reversal of promoter methylation.

The nutraceuticals-mediated epigenetic regulation of a number of cellular processes has been demonstrated in the recent years. This includes the epigenetic regulation of miRNAs and CSCs. As discussed in the next few sections, this is a rapidly emerging field with most of the reports appearing in last few years only. The general epigenetic regulation of genes by nutraceuticals has already been a topic of many available reviews. Thus, to keep our discussion very focused, we will only elaborate on the studies that have provided evidence, direct or indirect, on the ability of nutraceuticals to epigenetically regulate miRNAs and CSCs.

Epigenetic regulation of miRNAs

The involvement of miRNAs in regulation of cancer-related events is well established [53]. These small non-coding RNAs are now believed to be involved in every single aspect of the development of human cancers. Evidently, miRNAs are de-regulated in cancer cells with lowered expression of tumor suppressor miRNAs and an induction of oncogenic miRNAs, relative to normal tissue. Epigenetic changes seem to play an important role in such altered regulation of miRNAs in the cancer cells. In particular, aberrant DNA methylation and demethylation seems to play role in deregulation of specific miRNAs in various cancer types [54].

Epigenetic regulation of miRNAs by nutraceuticals

A number of reports, including some from our own laboratory, have documented the modulation of miRNAs by nutraceuticals [55-59]. Since epigenetic changes play a role in aberrant expression of several miRNAs in cancer cells, nutraceuticals are now being investigated for their ability to reverse these changes leading to restoration of 'normalcy' and the inhibition of tumor growth. In proof of the epigenetic regulation of miRNAs by dietary agents, diet-derived butyrate has been demonstrated to inhibit the oncogenic miR-17-92 cluster miRNAs by acting as a histone deacetylase inhibitor [60]. The histone deacetylase inhibition by butyrate was similar to well-known histone deacetylase inhibitors suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) which is indicative of a potent epigenetic regulation of miRNA by this dietary agent. Further, grape seed extract (GSE) has recently been shown to be effective against azoxymethane (AOM)-induced colon tumorigenesis in A/J mice [61, 62]. GSE administration for 18 to 28 weeks resulted in significantly reduced tumor burden. Along with the beneficial effects of GSE against NF-κB, β-catenin and MAPK signaling, there was evidence of GSE influencing the miRNA

expression profiles and miRNA processing machinery. It was suggested that the epigenetic modulation of miRNAs was responsible for the observed effects of GSE on inflammation, proliferation and apoptosis-induction [61].

There is evidence in support of epigenetic regulation by nutraceutical curcumin [63-66]. In an early report on the epigenetic regulation of miRNAs by this compound, this nutraceutical was reported to epigenetically modulate miR-203 in bladder cancer cells [67]. This study observed a significantly decreased expression of miR-203 in multiple bladder carcinoma cell lines such as T24, J82 and TCCSUP, when compared to normal immortalized uroepithelial cells SV-HUC-1 which suggested a tumor suppressive role of this miRNA in bladder cancer cells. Expression of miR-203 was also evaluated in human patient tissues and 11 out of 18 cases were observed to exhibit lowered expression as opposed to only 6 cases with increased expression. The cause of lowered expression of miR-203 in bladder cancer cells was determined to be its methylation in cancer cells. Treatment of bladder cancer cells with curcumin resulted in close to 2-fold increase in the expression of miR-203 which led authors to evaluate the effect of curcumin on methylation of this miRNA as a putative mechanism of action. Curcumin alone was found to partially demethylated miR-203 leading to the observed increase in expression. Consistent with miR-203 signaling, the target genes Akt2 and Src were found to be elevated in untreated bladder cancer cells but the demethylation and subsequent up-regulation of miR-203 by curcumin resulted in down-regulation of these two genes. The epigenetic regulation of miR-203 by curcumin resulted in reduced proliferation, invasion and an induction of apoptosis.

Isothiocyanates from cruciferous vegetables can affect the DNA methylation leading to their anticancer effects [68]. In addition to DNA methylation, isothiocyanate sulforaphane has also been shown to regulate histone deacetylase and miRNAs both alone [69] as well as in combination with another nutraceutical EGCG (epigallocatechin gallate) [70]. Dietary polyphenols have also been investigated for their anticancer activity for many years now [71-73] and more recent reports confirm the ability of this class of compounds to alter DNA methylation, histone modification and miRNAs [74].

In our laboratory, we observed an epigenetic regulation of miR-29a and miR-1256 by nutraceutical isoflavone [75]. The anticancer activity of isoflavones is well documented [76]. In support of the epigenetic regulation of miRNAs leading to their de-regulation, we found partial methylation of DNA sequence of miR-29a and miR-1256 in prostate cancer cells leading to their reduced expression. With the reduction in expression of these miRNAs, their target genes TRIM68 and PGK-1 were found to be induced in the prostate cancer cells. To test the epigenetic modulation of miR-29a and miR-1256 by isoflavone, if any, cells were treated with isoflavone and a demethylating effect of isoflavone on methylation sites in miR-29a and miR-1256 promoter sites was found. This clearly demonstrated an epigenetic regulation of miRNAs by isoflavone. Further, the demethylation of miR-29a and miR-1256 by isoflavone led to an abrogation of their inhibition and we observed an increased expression of these miRNAs with concomitant repression of their targets TRIM68 and PGK-1. These novel results on the epigenetic modulation of miRNAs by isoflavone opened the possibility of isoflavone being used as a non-toxic nutraceutical for reversal of DNA methylation.

BR-DIM (the specially formulated DIM with enhanced bioavailability) has been investigated for its anticancer effects for many years now. We recently observed an epigenetic regulation of miRNA by this nutraceutical. In a study to understand the miRNA regulation of androgen receptor (AR) signaling in castrate resistant prostate cancer (CRPC), we evaluated the expression of miR-34a in human patient samples [77]. This miRNA was shortlisted because of its ability to target AR. A reduced expression of miR-34a was observed in prostate cancer tissue specimens from patients with Gleason grade 7 and above, which was consistent with increased expression of AR. In mechanistic studies carried out in LNCaP and C4-2B cells, forced over-expression of miR-34a was found to result in reduced AR expression as well as reduction in the expression of another of its target – Notch1. These results suggested a regulation of AR and Notch 1 by miR-34a in prostate cancer cells. We evaluated BR-DIM intervention in prostate cancer patients and observed re-expression of miR-34a in patients who were administered BRDIM prior to radical prostatectomy. This observation suggested an effect of BR-DIM on miR-34a and made us question if the regulation of miR-34a by BR-DIM involved some epigenetic regulation. Treatment of LNCaP and C4-2B with 6μM BR-DIM for 5 days resulted in approximately 30% reduced methylation of miR-34a promoter in LNCaP cells and 40% reduced methylation in C4-2B cells. These results are a direct proof of epigenetic modulation of miR-34a by nutraceutical BR-DIM. Such epigenetic regulation by BR-DIM might be responsible for its eventual regulation of AR and Notch signaling in prostate cancer cells.

In summary, the data on epigenetic regulation of miRNAs by nutraceuticals is slowly, but surely, appearing (**Table 1**). This conclusion is based not only on *in vitro* observations, but also derives credibility from clinical samples. Going by the general trend, we should look forward to a spurt in such reports in next few years. Next, we evaluate the available data on epigenetic regulation of CSCs by nutraceuticals.

Epigenetic regulation of CSCs

The importance of CSCs in metastasis and drug resistance of human cancers is irrefutable [78, 79]. These specialized cells are endowed with very tight regulatory mechanisms to ensure their sustenance. With the overwhelming evidence supporting a role of epigenetics in virtually every disease, an epigenetic regulation of CSCs is also being appreciated [80]. Infact, it has been proposed that dynamic epigenetic changes in cancer cells / CSCs might facilitate an efficient inter-conversion of these cell types [81]. Simply speaking, this translates into an efficient epigenetic switch-on and switch-off of CSC markers within the tumor cells to derive either tumorigenic, drug-resistant CSCs or the rapidly dividing side-population cells. While such a theory sounds interesting, it still needs very convincing results for support. Irrespective of the resident CSC theory, it is beyond doubt that the agents capable of targeting CSCs stand a better chance as cancer therapeutic options in the clinical management of human cancers. In the next section we discuss the emerging literature that showcases epigenetic regulation of CSCs by nutraceuticals.

Epigenetic regulation of CSCs by nutraceuticals

As opposed to epigenetic regulation of miRNAs by nutraceuticals, which itself is an emerging field, the reports on epigenetic regulation of CSCs by nutraceuticals are even scarce. There are a number of available reports that suggest regulation of CSCs by nutraceuticals [82-86]. However, it's the mechanism of such regulation, namely epigenetic, which makes the topic challenging. In this section we attempt to make a connection between the epigenetic activity of nutraceuticals and the inhibition of CSCs.

EZH2 is an important epigenetic regulator of CSC function. It is a histone methyltransferase enhancer of polycomb group complexes involved in epigenetic regulation [87]. In a study conducted in pancreatic cancer cells, we found increased expression of EZH2 in tumor sphere cells derived from MiaPaCa-2 pancreatic cancer cells [88]. An increased expression of multiple CSC markers, such as EpCAM, lin28B and nanog was also observed in these cells which suggested that these sphere cells (pancreatospheres) were enriched for CSC markers. With the known epigenetic functions of EZH2, we investigated the effect of our novel curcumin analog CDF (curcumin difluorinated) on this factor. In pancreatospheres, CDF significantly down-regulated the mRNA expression of EZH2. Since EZH2 mediates epigenetic histone modifications that are critical for CSC functions [89], our results are suggestive of an ability of CDF to epigenetically regulate CSCs. This assumption is further supported by the observed inhibition of pancreatospheres by CDF and the reduced levels of EZH2 in tumor remnants of CDF-treated mice in which pancreatic cells-derived tumors were established orthotopically. As expected, reduced EZH2 levels correlated with the reduced CSC markers *in vitro* as well as *in vivo*. Such down-regulation of EZH2 by CDF's parent compound curcumin has been demonstrated in breast cancer cells [90] indicating an epigenetic regulation of CSCs by this class of compounds that needs to be explored further. EZH2 expression has also been reported to be down-regulated by nutraceuticals EGCG [91, 92], BR-DIM [93] and sulforaphane [94].

A number of CSC markers are being pursued in modern day cancer research and these vary a lot, depending on specific cancer type. However, as evident from discussion above, a direct epigenetic regulation of CSC markers by nutraceuticals is yet to be demonstrated. Down-regulation of EZH2 is an indirect epigenetic regulation of CSCs. Another indirect connection can be made through epigenetic regulation of EMT by nutraceuticals. The EMT-CSC nexus is a hot topic and recently EGCG was reported to inhibit histone deacetylase activity in pancreatic cancer cells resulting in reversal of EMT through up-regulation of epithelial marker e-cadherin and inhibition of NF-κB, snail and the MMPs (matrix metalloproteinases) [95]. EGCG inhibits multiple enzymes involved in epigenetic regulation, resulting in reduction of 5-methylcytosine and methylation levels, leading to de-repression of tumor suppressive p21 [96].

DNMT1, the DNA (cytosine 5)-methyltransferase enzyme, is an important regulator of epigenetic changes. It transfers methyl groups onto the cytosine residues in the promoter regions of target miRNAs thereby suppressing their expression. A complex relationship between DNMT1 and the CSC markers oct4, sox2 and nanog2 has been demonstrated that also includes regulation through miR-302 [97]. DNMT1 also plays a role in the miR-29a-

mediated EMT [98]. All of these evidences indicate a vicious connection between DNMT1 and the epigenetic regulation of EMT-CSC nexus. Not surprisingly, a number of nutraceuticals can inhibit DNMT1 [45, 96, 99-101] which further provides credibility to the hypothesis that nutraceuticals possess the potential to epigenetically regulate both miRNAs and CSCs.

Conclusions and perspectives

In this article, we have summarized the literature on the epigenetic modulation of miRNAs and CSCs by various nutraceuticals (**Fig 1**). This is an emerging area of research and clearly there are many well-known nutraceuticals that have been investigated for chemopreventive/therapeutic action but not yet demonstrated to epigenetically regulate miRNAs/CSCs. This does not necessarily mean that those nutraceuticals lack such activity. As referenced above, a number of nutraceuticals have been shown to epigenetically regulate some genes/factors and, additionally, there are reports on general activity of nutraceuticals against the enzymes involved in epigenetic regulation [45]. All this seems to suggest that it's just a matter of time before reports on the epigenetic regulation of miRNAs and CSCs by more nutraceuticals start trickling in. Nutraceuticals possess another important trait that adds to their anticancer appeal. They are pleiotropic i.e. a single agent can modulate multiple signaling pathways. This is clearly evident from the discussion above where the same nutraceutical might have been discussed at different places in context of its ability to modulate different factors leading to the epigenetic regulation. Since cancer cells frequently switch their dependency on specific signaling pathways, particularly in the presence of targeted drugs, being pleiotropic offers a distinct advantage to nutraceuticals. This activity of nutraceuticals has been demonstrated over and over again in pre-clinical studies but is yet to be demonstrated in a successful clinical trial.

In addition to the role of epigenetics in progression of human cancers, an interesting role of epigenetics in cancer health disparities has also been proposed [102]. This stems from the observations that there are significant epigenetic differences among racial and ethnic groups. Such epigenetic differences might be pertinent to general aggressiveness and outcome of various cancers in the human sub-populations. This particular field of epigenetics research holds a lot of promise because health disparities in cancer remain one of the least understood topics. Epidemiological data have linked specific nutraceuticals to reduced incidence(s) of various cancers. It will be interesting to evaluate if some underlying epigenetic regulation(s) by nutraceuticals in various ethnic/regional populations deserve credit for this. Epigenetics is an emerging field not without its own share of controversies [103, 104]. The challenge, moving forward, is in finding a way to apply all the available information for the benefit of numerous patients battling with this deadly disease.

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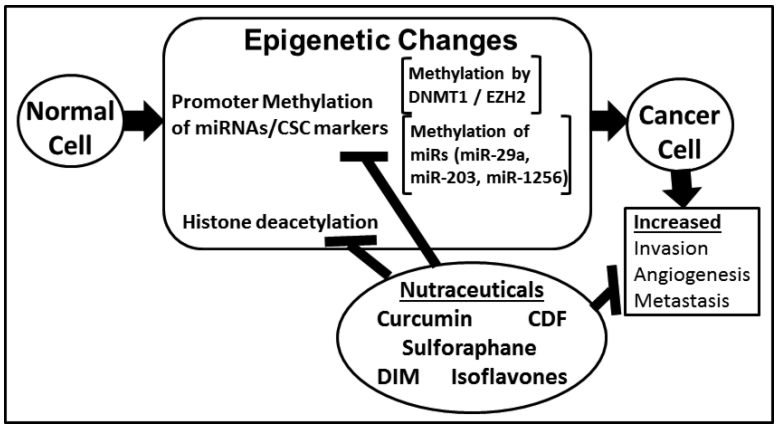

Figure 1. Epigenetic regulation by nutraceuticals

A number of DNA and Histone modifications constitute epigenetic changes that regulate gene expression. These include histone deacetylations and promoter methylations of miRNAs / CSCs leading to transformation of normal cells into cancer cells with induced invasion, angiogenesis and metastasis. . Nutraceuticals can reverse epigenetic changes by inhibiting DNMT1/EZH2, the methyltransferases that play a role in CSC enrichment. Nutraceuticals can also reverse methylation of miRNAs as evident by reports on miRs-29a, 203 and 1256. Such reversal of epigenetic modifications results in induction of apoptosis, reduced invasion/metastasis, and may explain the anticancer effect of these nutraceuticals.

Table 1

miRNAs that are epigenetically regulated by nutraceuticals

Nutraceutical	Target miRNA	Reference
Curcumin	miR-203	[67]
Dietary butyrate	miR-17-92 cluster	[60]
DIM (BR-DIM)	miR-34a	[77]
Grape seed extract	General miRNA expression	[61]
Isoflavone	miR-29a	[75]
Isoflavone	miR-1256	[75]

A number of nutraceuticals are known to regulate miRNA expression leading to their anticancer effects. However, the epigenetic regulation of miRNAs by nutraceuticals has only been studied in a few studies listed here.