A possible link between specific transfer RNA methylation and tumorigenic phenotype of breast cancer

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The human RNA methyltransferase BCDIN3D is overexpressed in breast cancer cells and involved in cellular invasion and poor prognosis of breast cancer. Several years ago, BCDIN3D was reported to dimethylate the 5'-monophosphate of specific precursor miRNAs (pre-miRNAs), such as the tumor suppressor miR145. Dimethylation of the 5'-monophosphate of the pre-miRNA negatively regulates the subsequent processing by Dicer in vitro, and results in the downregulated expression of the mature form of the miRNA. The depletion of BCDIN3D also reportedly results in the suppression of the tumorigenic phenotype of breast cancer cells. Thus, these findings suggested that BCDIN3D promotes the cellular invasion of breast cancer cells, by downregulating the expression of tumor suppressor miRNAs via the dimethylation of the 5'-monophosphate of the corresponding pre-miRNAs. Recently, we found that cytoplasmic tRNA^His is actually the primary target of human BCDIN3D, rather than pre-miR145. BCDIN3D monomethylates the 5'-phosphate of cytoplasmic tRNA^His much more efficiently than that of pre-miRNA in vitro, and is responsible for the monomethylation of the 5'-phosphate of cytoplasmic tRNA^His in vivo. BCDIN3D recognizes the eight-nucleotide long extended acceptor helix with the G_1-A_73 mis-pair at the top of the acceptor stem of tRNA^His, which are exceptional features among cytoplasmic tRNA species. These results not only reveal the primary target of BCDIN3D, which is overexpressed in breast cancer cells, but also highlight the possible involvement of the 5'-phosphomethylation of tRNA and/or tRNA in the tumorigenesis of breast cancer cells, beyond its established function in protein synthesis.

Keywords: cytoplasmic tRNA^His; methylation; breast cancer; BCDIN3D

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BCDIN3D, the BCDIN3 (bicoid-interacting protein 3) Domain containing protein, contains an S-(5'-adenosyl)-L-methionine (AdoMet) binding motif, and is homologous to a conserved family of eukaryotic protein methyltransferases acting on RNA-binding proteins [1]. BCDIN3D is conserved from worm to human [2]. The BCDIN3D mRNA is overexpressed in human breast cancer cells, and the elevated expression of BCDIN3D is related to poor prognosis in breast cancer [3]. The biological roles and functions of BCDIN3D are poorly understood, and the molecular basis of the involvement of BCDIN3D in the tumorigenic phenotype of breast cancer has remained enigmatic.
Several years ago, it was reported that BCDIN3D dimethylates the 5'-monophosphate of specific precursor micro RNAs (pre-miRNAs), such as the tumor suppressors miR145 and miR23b \[^2, 4-7^\] . Dimethylation of the 5'-monophosphate of the pre-miRNA negatively regulates the subsequent processing by Dicer \textit{in vitro}, and results in the downregulated expression of the mature miRNA. The downregulation of tumor suppressor miRNAs could be related to the tumorigenic phenotype of breast cancer cells. Indeed, it was also reported that the depletion of BCDIN3D resulted in the suppression of the tumorigenic phenotype of MDA-MB231 breast cancer cells \[^2^\]. Therefore, it was suggested that BCDIN3D promotes the cellular invasion of breast cancer cells, by negatively regulating the expression of tumor suppressor miRNAs. However, the mechanisms by which BCDIN3D recognizes only a specific group of pre-miRNAs and negatively regulates the expression of mature miRNAs in breast cancer cells are not well understood.

With an aim to identify other possible RNA substrates that are methylated by BCDIN3D, and to clarify the mechanism by which BCDIN3D recognizes specific RNAs and controls their expression, we analyzed BCDIN3D-binding RNAs in human HEK293T cells \[^5^\]. When BCDIN3D, expressed in HEK293T cells, was purified from the cell extract, we noticed that a distinct, seventy-eighty nucleotide RNA molecule was co-purified with the BCDIN3D protein. Since the nucleotide sequences of cytoplasmic tRNA\textsuperscript{His} from human and fruit fly were reported to have a 5’-monomethylphosphate \[^9, 10^\], we assumed that the RNA might be cytoplasmic tRNA\textsuperscript{His}. The analysis of the RNA co-purified with BCDIN3D, by RT-PCR and DNA sequencing of RT-PCR products, confirmed that cytoplasmic tRNA\textsuperscript{His} co-purified with BCDIN3D from the cell extracts, but other tRNAs, such as tRNA\textsuperscript{Phe}, did not. A subsequent detailed analysis of the RNaseT\textsubscript{1} and RNaseA-digested fragments of the RNA by LC-mass spectrometry \[^11-13^\] clearly demonstrated that the RNA is cytoplasmic tRNA\textsuperscript{His}. More importantly, the 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His} was monomethylated, but not dimethylated. The 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His}, co-purified with BCDIN3D from extracts of HEK293T cells overexpressing BCDIN3D, is fully methylated. Thus, we asked whether the 5’-phosphate of cytoplasmic tRNA\textsuperscript{His} is methylated under normal physiological conditions. Cytoplasmic tRNA\textsuperscript{His} was purified from HEK293T cells and analyzed by LC-mass spectrometry, which revealed that the 5’-monophosphate of tRNA\textsuperscript{His} is fully monomethylated under normal physiological conditions in HEK293T cells.

These results prompted us to examine whether BCDIN3D could methylate the 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His}. The recombinant human BCDIN3D protein was expressed in \textit{Escherichia coli}, and its enzymatic activity was examined using the human cytoplasmic tRNA\textsuperscript{His} transcript as the substrate and SAM [S-(5’-adenosyl)-L-methionine] as the methyl-group donor \textit{in vitro}. The results showed that the cytoplasmic tRNA\textsuperscript{His} transcript is efficiently methylated by BCDIN3D \textit{in vitro}. However, unexpectedly, the human miR145 precursor transcript, which was previously shown to be dimethylated by BCDIN3D, is hardly methylated under the same conditions. The reaction products were further analyzed by LC-mass spectrometry, and the results confirmed that BCDIN3D monomethylates the 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His} much more efficiently than the pre-miR145 transcript. Moreover, BCDIN3D could not dimethylate either tRNA\textsuperscript{His} or pre-miR145 \textit{in vitro}. The steady state kinetics of the methylation of these RNA substrates also confirmed that cytoplasmic tRNA\textsuperscript{His} is a better substrate than pre-miR145, by over two to three orders of magnitude.

To explore whether BCDIN3D is responsible for the monomethylation of the 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His} \textit{in vivo}, BCDIN3D-knockdown cells were established from HEK293T cells by CRISPR/Cas9 editing \[^8^\]. The growth rate of the BCDIN3D knockdown cells was slightly slower than that of the parental HEK293T cells. The cytoplasmic tRNA\textsuperscript{His} isolated from the BCDIN3D knockdown cells lacked the methyl moiety at its 5’-monophosphate. The exogenous expression of BCDIN3D in the BCDIN3D knockdown cells restored the 5’-monomethylphosphate modification of the cytoplasmic tRNA\textsuperscript{His}. Thus, BCDIN3D catalyzes the monomethylation of the 5’-monophosphate of cytoplasmic tRNA\textsuperscript{His} in HEK293T cells, under normal physiological conditions. Moreover, except for cytoplasmic tRNA\textsuperscript{His}, no other RNAs in BCDIN3D knockdown cells are significantly methylated by recombinant BCDIN3D \textit{in vitro}. Together with the results obtained in the \textit{in vitro} methylation assay, using recombinant BCDIN3D and the tRNA\textsuperscript{His} transcript, it is most likely that the primary target of BCDIN3D is cytoplasmic tRNA\textsuperscript{His} rather than pre-miRNAs, and BCDIN3D has a monomethylation activity acting on the 5’-phosphate of RNA \[^8^\]. Under specific conditions or in certain biological processes, the pre-miRNA might be methylated efficiently, and unknown regulatory factors specific to breast cancer cells might enhance the efficient pre-miRNA (di)methylation process \textit{in vivo}. The \textit{in vivo} mechanism of the dimethylation of the 5’-monophosphate of specific pre-miRNAs, such as pre-miR145, in breast cancer cells awaits further studies.

How does BCDIN3D specifically recognize cytoplasmic tRNA\textsuperscript{His}? Human cytoplasmic tRNA\textsuperscript{His} is matured through unique processes \[^17, 18^\]. After transcription by RNA
polymerase-III, the 5'-leader and 3'-tail sequences of the precursor tRNA\textsubscript{His} are removed. Subsequently, a single guanosine residue (G) is attached to the 5'-end (at position -1; G\textsubscript{-1}) by a tRNA\textsubscript{His}-specific guanylyltransferase (Thg1) \cite{17, 19, 20} and the CCA is added at the 3'-end (positions 74-76) \cite{21}. As a result, the mature cytoplasmic tRNA\textsubscript{His} has an eight-nucleotide long acceptor helix with G-1-A\textsubscript{73} mis-pairing at the top of the helix, while other cytoplasmic tRNAs have seven-nucleotide long acceptor helices. Analyses of the \textit{in vitro} steady state kinetics of methylation of mutant cytoplasmic tRNA\textsubscript{His} transcripts by recombinant BCDIN3D revealed that BCDIN3D recognizes G\textsubscript{-1}, and the eight-nucleotide long extended acceptor helix with G\textsubscript{-1-}A\textsubscript{73} mis-pairing at the top of the acceptor stem of cytoplasmic tRNA\textsubscript{His}. Thus, BCDIN3D recognizes the unique features of cytoplasmic tRNA\textsubscript{His}, and discriminates cytoplasmic tRNA\textsubscript{His} from other tRNA species \cite{8}.

The function of the 5'-monomethylphosphate of cytoplasmic tRNA\textsubscript{His} has remained elusive. While the 5'-monomethylphosphate of cytoplasmic tRNA\textsubscript{His} lowers the affinity of tRNA\textsubscript{His} toward histidyl-tRNA synthetase (HRS), as expected from the complex structure of bacterial HRS with tRNA\textsubscript{His} \cite{22}, the overall aminoacylation efficiency is not affected by the modification. The steady-state levels of cytoplasmic tRNA\textsubscript{His} in parental HEK293T and BCDIN3D-knockdown cells are not significantly different. Furthermore, the stabilities of cytoplasmic tRNA\textsubscript{His} from HEK293T and BCDIN3D-knockdown cells after the treatment of the cells with actinomycin-D were not significantly different. However, the 5'-monomethylmonophosphate protects cytoplasmic tRNA\textsubscript{His} from degradation \textit{in vitro}, in cytoplasmic cell extracts. Thus, BCDIN3D could act as a cytoplasmic tRNA\textsubscript{His}-specific 5'-methylphosphate capping enzyme, and the methylation of the 5'-monophosphate of cytoplasmic tRNA\textsubscript{His} might be

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**Figure 1. A possible link between tRNA methylation and the tumorigenic phenotype of breast cancer.** BCDIN3D is overexpressed in breast cancer cells, and the elevated expression is related to the tumorigenic phenotype and poor prognosis of breast cancer. Cytoplasmic tRNA\textsubscript{His} is now found to be the primary target of BCDIN3D. The 5'-monophosphate of cytoplasmic tRNA\textsubscript{His} is monomethylated by BCDIN3D. The modification of tRNA\textsubscript{His} and/or tRNA\textsubscript{His} itself might be involved in the tumorigenic phenotype of breast cancer, beyond its established function in protein synthesis.
involved in increasing its stability under specific conditions or in certain biological processes.

The elucidation of the involvement of the 5'-monophosphate methylation of cytoplasmic tRNA\(^{\text{His}}\) in the tumorigenic phenotype of breast cancer is challenging (Figure 1). tRNAs are classical non-coding RNAs, and their established functions, as adaptor molecules between genetic codes and amino acids, have been well studied for more than sixty years. However, besides their roles as adaptor molecules in protein synthesis, tRNAs are involved in various biological processes in cells \[23-25\]. Recent studies have shown that the expression level of initiator tRNA\(^{\text{Met}}\) is elevated in breast cancer cells \[26\]. The upregulation of specific tRNAs, such as tRNA\(^{\text{Glu}}\)UUC and tRNA\(^{\text{Arg}}\)CCG, reportedly stabilizes mRNAs containing the corresponding codons and enhances translation in highly metastatic breast cancer cells \[27\]. However, the steady state level of cytoplasmic tRNA\(^{\text{His}}\) is not affected by the knockdown of BCDIN3D in HEK293T cells. Thus, it is unlikely that the methylation of the 5'-monophosphate of cytoplasmic tRNA\(^{\text{His}}\) increases the translation of specific mRNAs, although it should be examined in the future. Recent studies also have provided evidence that small RNA fragments derived from tRNAs - tRNA fragments (tRFs) - participate in various cellular functions \[23-25\]. These tRFs are often produced under stress conditions \[28-32\]. In breast and prostate cancers, specific tRNAs, such as cytoplasmic tRNA\(^{\text{Asp}}\) and tRNA\(^{\text{His}}\), are cleaved by angiogenin, and the tRNA halves are abundantly expressed in a sex hormone-dependent manner \[33\]. These tRNA halves have also been shown to promote the proliferation of breast and prostate cancer cells, by an as yet unknown mechanism. In human and mouse cells, 3'- or 5'-terminal tRFs (3'-tRF or 5'-tRF) reportedly accumulate in cells in an asymmetric manner, and these tRFs associate with Ago2 \[34\]. These tRFs probably act as typical miRNAs. tRNA modifications, such as 5-methyl cytidine, are associated with the stress induced cleavage of tRNA molecules and the production of tRFs in cells \[31\]. The 5'-monomethylation of the 5'-monophosphate of tRNA\(^{\text{His}}\) may control the expression of the tRNA halves and/or tRFs derived from tRNA\(^{\text{His}}\) in breast cancer cells or under specific biological or stress conditions. Future work will clarify whether the methylation of the 5'-phosphate of tRNA\(^{\text{His}}\) by BCDIN3D is involved in the tumorigenic phenotype of breast cancer and other cancers.

**Conflicting interests**

The authors have declared that no conflict of interests exist.

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