RNA Modifications

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Parallel Isotope-Based Quantification of Modified tRNA Nucleosides**

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Transfer RNAs (tRNAs) are the essential adapter molecules needed for the translation of the genetic code into a peptide sequence at the ribosome. Human cells possess 49 different isoacceptor tRNAs, each carrying a specific amino acid at the 3′ terminus.[1] All tRNA molecules consist of 70–100 ribonucleotides of which up to 20% are modified. The large number (>95) of different modifications known today[2,3] and in particular their structural diversity establish a second layer of molecular information most likely required for the decoding process.[4] However, only limited information is available as to how these modifications influence the translation of genetic information,[5] the folding of tRNA,[4,6] and tRNA function.[7]

In the current era of "omics" research the central scientific approach is the study of cellular components not individually but context based.[8] In our opinion, a new quantitative method is also needed for the analysis of modified tRNA nucleosides.[3,9] As the literature indicates that tumor cells possess a significantly different tRNA modification pattern than that in normal cells,[10,11] particularly in their mitochondria,[12] such a method would lead to a better characterization of tumor cells and may enable deeper insight in the metabolism of tumor cells.[11] Here, we report an isotope-based, mass-spectrometric method that allows direct, parallel quantification of in principle all tRNA modifications with extremely high sensitivity. We provide initial data showing that surprisingly large variations are observed among tumor cells and also between tumor cells and non-tumorogenic tissue.

The method is represented in Figure 1. Whole tRNA is isolated from cells or tissue using a modified literature protocol.[13] The bulk tRNA material is subsequently fully digested to obtain a mixture of the tRNA nucleosides.[14] In order to quantify certain modifications, we determined calibration curves for each modification individually and added precisely known amounts of synthetic isotope-labeled, modified tRNA nucleosides to the digests. To develop the method we first attempted to quantify the tRNA modifications m^6A, m^2A, Am, t^6A, i^6A, and ms^2i^6A,[15] because these

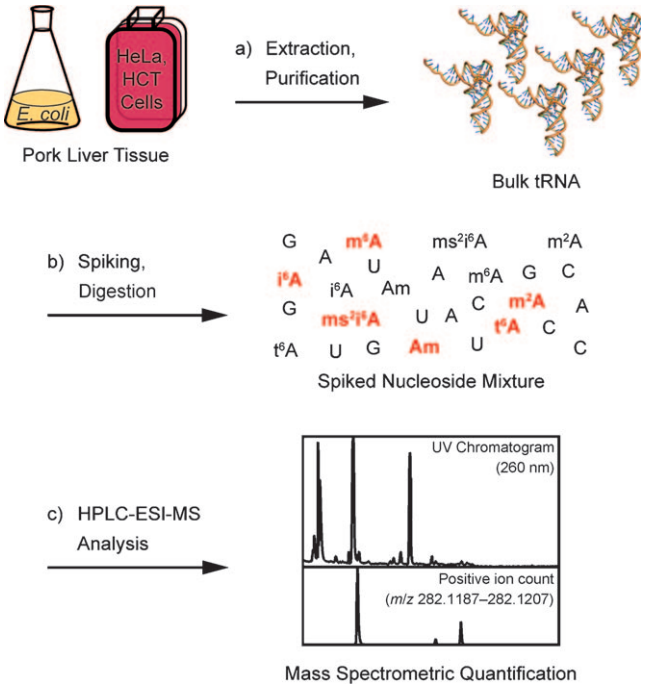

Figure 1. Depiction of the quantitative method for analysis of modified tRNA nucleosides. a) Extraction: phenol extraction; purification: PD10 chromatography, anion-exchange chromatography; b) Spiking: addition of isotope-labeled standards; Digestion: enzymatic digestion of the bulk tRNA mixture; black symbols show natural nucleosides; red symbols indicate added isotope-labeled nucleosides; c) HPLC–ESI-MS analysis: chromatographic separation and mass spectrometric analysis (for experimental details see the Supporting Information).

nucleosides are mostly present directly 3′-adjacent to the anticodon[2,3] and therefore likely to be directly involved in the decoding process.[16] The nucleoside ms^2i^6A is a particularly interesting modification because it exists only in prokaryotes and mitochondrial tRNAs.[2,3] As such, study of this nucleoside may allow insight into the mitochondrial activity of cells. All mentioned modified nucleosides were synthesized as isotope-labeled (*) derivatives as indicated in Figure 2.[17] The digest mixture containing the added isotope-labeled compounds was subsequently analyzed by HPLC–ESI-MS.

Typical UV traces of the obtained *E. coli* and pork liver modification pattern are depicted in Figure 3a and 3b, respectively. Next to the dominant signals for the canonical RNA nucleosides, the various modified nucleosides either appear as small signals or are hidden in the base line. The modifications were assigned unambiguously by MS analysis. In Figure 3c the ion counts of m^2A and m^6A in comparison to

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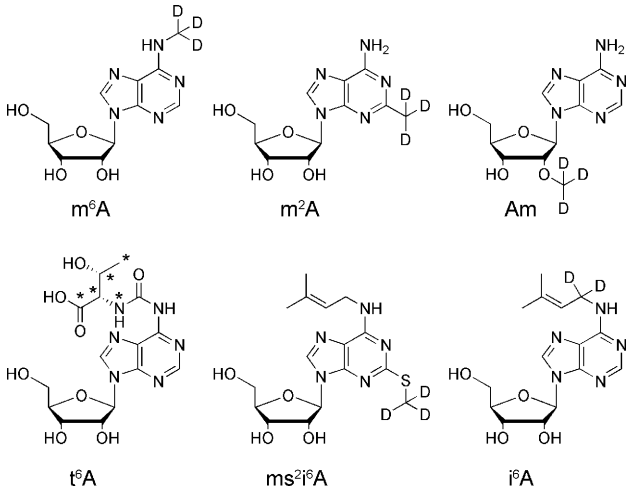

Figure 2. Heavy-atom-labeled adenosine modifications investigated. Asterisks (*) are used to label ^{15}N and ^{13}C atoms; D = deuterium. Base-modified nucleosides occur 3′-adjacent to the anticodon in position 37. The ribose-modified nucleoside Am is present in position 4.

the added synthetic isotope-labeled species are depicted as an example. Because of the mass difference they appear in the mass spectra as two separate signals allowing quantification by integration of the area under the mass peaks. The high quality of the obtained data with an average error margin of only 5% proves that the isotope dilution method can be readily applied for the analysis of tRNA modifications if isotope-labeled, modified RNA nucleosides are available.[18]

With the new method for tRNA analysis in hand we started to investigate how the modification patterns of tumor cells differ from each other and from healthy tissue. For an initial screening we used the human epithelial cell lines HCT-116 and HeLa, which were derived from a colorectal tumor and from an adenocarcinoma of the cervix, respectively. For comparison, we also analyzed the modification pattern of pork liver and *E. coli*. The results of the determinations are depicted in Figure 4. In agreement with literature data we detect the modification m^2A only in *E. coli* and not in eukaryotic cells. In contrast, the Am modification[19] is found only in eukaryotic cells.[2,3,20] To our surprise we observed that the level of t^6A in healthy pork liver is significantly lower than that in both tumor cell lines, which indicates that the biosynthesis of this modification may be upregulated in HeLa and HCT-116 cells. Further significant differences between the tumor cell lines were noted for m^6A and i^6A. HeLa cells contain twice as much m^6A than HCT-116 cells, which exhibit the same amount of m^6A as pork liver tissue. The level of i^6A is 30% lower in HeLa cells than in HCT-116 cells, whereas HeLa cells contain the same amount of i^6A as healthy liver tissue. These results support the idea that the analysis of modified tRNA nucleosides can differentiate between tumorogenic and non-tumorogenic cells and that even discrimination of different cancer cell lines may become possible once the analysis is extended to a broader variety of nucleoside modifications.

We next addressed the question of how the mitochondrial tRNA modification pattern changes in tumor cells. It has been

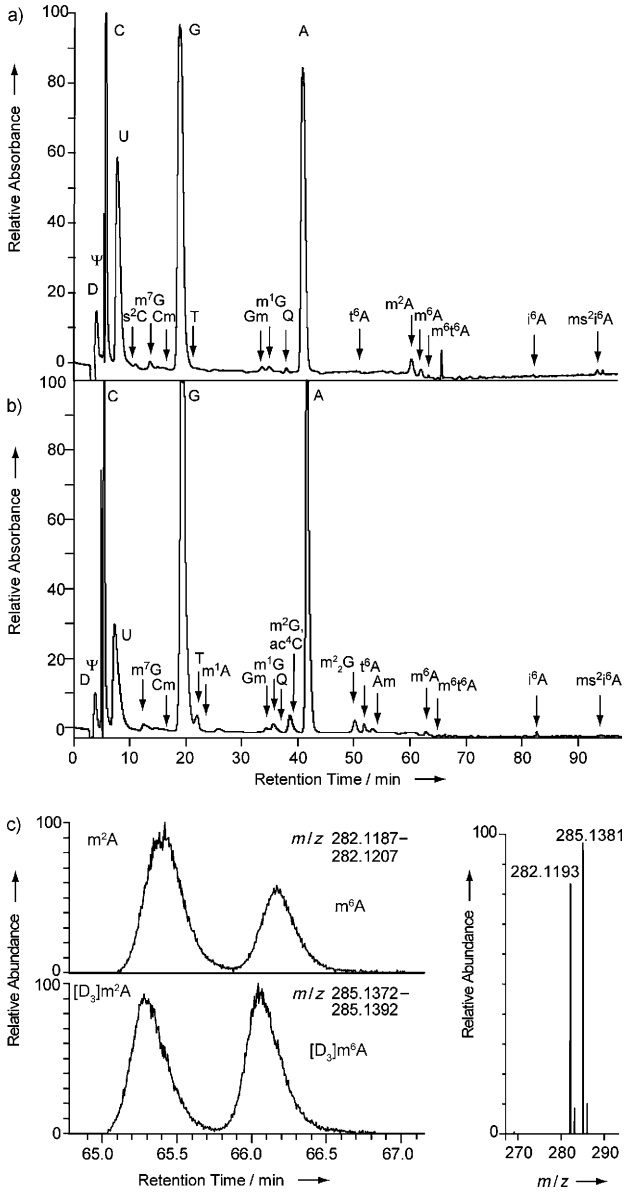

Figure 3. a) HPLC trace of the tRNA digest from *E. coli* ($\lambda = 260$ nm). b) HPLC trace of the tRNA digest from pork liver ($\lambda = 260$ nm). c) Positive ion traces of the protonated nucleosides m^2A and m^6A and the corresponding synthetic isotope-labeled derivatives (left). Relevant high-resolution mass spectrometric data for unlabeled and labeled m^2A (right).

known for some time that tumors have a reduced oxidative phosphorylation activity and that many tumor cells derive most of their needed energy from glycolysis,[21,22] which induces a reduced pH value in tumor tissue.[21] In order to investigate this phenomenon we used the isotope method for quantification of ms^2i^6A, which is present only in mitochondria of eukaryotic cells.

The result of this analysis is depicted in Figure 4 (also see inset). As expected, the modification ms^2i^6A is present in the bacterium *E. coli*. In addition, it is detectable in small but significant amounts in pork liver. The ability to quantify mitochondrial ms^2i^6A proves again the high sensitivity of the isotope-based method.

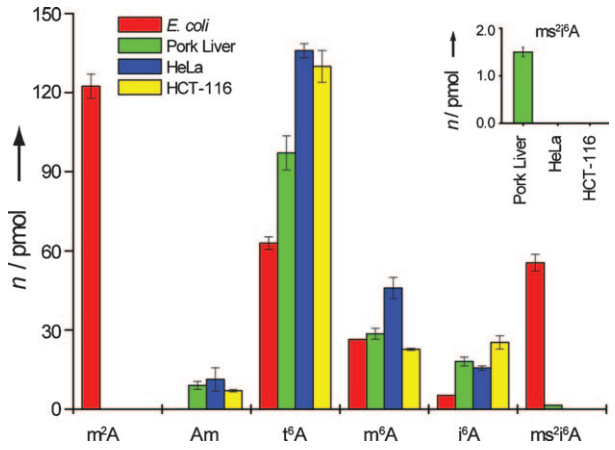

Figure 4. Comparison of modified nucleoside levels of *E. coli*, pork liver, HeLa, and HCT-116 cell lines. Error bars represent the standard deviations calculated from multiple experiments.

To our surprise we found that the analyzed tumor cells do not contain the modification ms^2i^6A at all, indicating that the mitochondrial tRNAs in tumor cells and normal cells indeed differ substantially. This result provides a first indication that the impairment of mitochondria in tumor cells (Warburg hypothesis)[23] is detectable using our isotope-based analysis method.

Even though our interpretation is currently based on the analysis of only six tRNA modifications, the results clearly show that the reported isotope-labeling method can rapidly provide quantitative data about tRNA modification levels in cells and tissues. Even quantification of modifications present only in mitochondrial tRNA is possible. Assuming that different species, various mammalian tissues, and different tumor tissues possess substantially different patterns of modified tRNA nucleosides,[24] then this isotope method opens new doors for the detailed analysis of cells and tissues. The presented method, for which we suggest the name "modiomics", might pave the way for the detailed analysis of the function and biosynthesis of modified tRNA nucleosides.

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[15] Abbreviations: m^6A: N^6-methyladenosine, m^2A: 2-methyladenosine, Am: 2′-*O*-methyladenosine, t^6A: N^6-threonylcarbamoyladenosine, ms^2i^6A: 2-methylthio-N^6-isopentenyladenosine, i^6A: N^6-isopentenyladenosine.
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