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Review

Ex-translational function of tRNAs and their fragments in cancer

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Transfer RNA (tRNA) molecules are most commonly known as the molecular amino acids carriers and also because of the role they play in a protein biosynthesis process. However, tRNA biology has revealed stupendous levels of many unexpected discoveries that put a new light on tRNA function in different processes besides translation, like apoptosis or cancer development. In recent years various species of RNAs have been found differentially expressed in different types of cancer. In this review we focus our attention on tRNAs as well as on tRNA-derived small RNAs ex-translational functions in human cells in oncogenesis and oncobiology.

Key words: tRNA, tRNA-derived fragments, cancer

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INTRODUCTION

Cancer is a multistep genetic and epigenetic disease with a complex etiology. Several defects such as mutations, down-regulation, over-expression and deletions in oncogenes and tumor suppressor protein-coding genes have been extensively described in cancer cells (Loeb *et al*., 2003; Sung *et al*., 2003). Recently, transcriptome analysis and different experimental approaches are providing strong evidence that also defects in non-protein coding RNAs (ncRNAs) might occur in tumors (Sana *et al*., 2012). microRNAs could serve as an excellent example of small ncRNAs, which expression is specifically regulated in cancer cells: e.g. mir-143 and mir-145 are down-regulated in colon cancer (Michael *et al*., 2003), mir-155/BIC is overexpressed in Burkitt and B cell lymphomas (Metzler *et al*., 2004; Eis *et al*., 2005). It is also well-known that in both plants and animals, some small RNAs are able to travel between tissues within an organism, thus transferring their functions to other cells (Dinger *et al*., 2008; Melnyk *et al*., 2011). In vertebrates, there has been much interest in the presence of specific short RNAs in the plasma and serum (Noerholm *et al*., 2012; Tsui *et al*., 2006). There is some evidence that short RNAs could be taken up by cells and alter gene expression, and it might be also interesting that possibly they can represent biomarkers of predisposition to specific diseases, including cancer (Hauptman *et al*., 2013). In this review we focus on the one group of non-protein coding RNAs: tRNAs as well as tRNA-derived small RNAs and their involvement in cancer.

TRANSFER RNAs

Up to 506 genes encoding a set of 49 different tRNAs are found in the human genome (http://lowelab.ucsc.edu/GtRNAdb/Hsapi). tRNA genes are transcribed by RNA polymerase III as pre-tRNAs in the nucleus. Pre-tRNA transcripts have the typical clover leaf structure with additional 5'-leader and 3'-trailer sequences of various lengths. Some pre-tRNAs contain intronic sequences (14–60 nt in length) positioned 3' to the anticodon that are removed by the evolutionarily conserved tRNA splicing endonuclease and the tRNA splicing ligase complex (Ableson *et al*., 1998). The 5' and 3' ends are trimmed by the endonucleases RNase P (Frank *et al*., 1998) and RNase Z (Ceballos *et al*., 2007), respectively.

After removal of the 3'-trailer, the terminal trinucleotide 5'-CCA-3', obligatory for the aminoacylation of tRNAs, is added at the 3'-acceptor stem by mitochondrial tRNA-nucleotidyltransferase 1 (TRNT1), as the CCA is not genome-encoded in eukaryotes. During the process of maturation, tRNA undergoes many modification events to generate non-canonical bases. About 10 percent of the bases in pre-tRNAs are modified enzymatically. Three types of base modifications occur: replacement of U residues at the 3' end of pre-tRNA with a CCA sequence, which is found at the 3' end of all tRNAs; addition of methyl and isopentenyl groups to the heterocyclic ring of purine bases and methylation of the 2'-OH group in the ribose of any residue; and conversion of specific uridines to dihydrouridine, pseudouridine, or ribothymidine residues. Modifications are involved in ensuring the correct secondary and tertiary structures (Durdevic *et al*., 2013). Finally, tRNAs are exported from the nucleus to become a competent substrate for translation. For its main function, each tRNA is charged with an amino acid that is covalently linked to the adenosine of the 3'CCA end, a reaction that is performed by cognate aminoacyl-tRNA synthetases. By reading the mRNA three nucleotides at a time by base-paring, the codon (mRNA)–antidocon (tRNA) interaction defines the amino acid position in the protein (Giege 2008). On the ribosome, tRNAs do not solely serve as passive substrate for amino acid polymerization, but provide a functional group (the ribose 2' hydroxyl at the 3' terminal adenosine) that has been proposed to directly participate in the chemistry of peptide bond formation (Dorner *et al*., 2002).

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Abbreviations: tRNA, transfer RNA; ncRNA, non-protein coding RNA; miRNA, microRNA; RNA Pol III, RNA polymerase III; nt, nucleotide; eIF2, eukaryotic initiation factor 2; tRF, tRNA-derived fragment; tiRNA, stress-induced small RNA; TRNT1, tRNA-nucleotidyltransferase; tsRNA, tRNA-derived small RNA; ubcRNA, urinary bladder carcinoma RNA; Ago, Argonaute protein; SITS, sense-induced transgene silencing

Microarray results show overall variations in the expression levels of tRNA among different tissues (Dittmar *et al*., 2006). For example, all nuclear-encoded tRNAs in ovary have lower levels relative to brain. Some tRNAs in spleen have higher (e.g. Phe-GAA, Met^i, Tyr-GTA, Trp-CCA), while others have lower (e.g. Leu-TAA, Glu-YTC) levels, compared to those in brain. Within individual tissues, the maximal differences between the relative tRNA levels can be as large as approximately tenfold (e.g. vulva, thymus) or only approximately threefold (e.g. testis). These findings demonstrated the existence of tissue-specific expression of tRNA species and strongly implicated the role for tRNA heterogeneity in regulating translation.

tRNAs play roles also outside of their main function in protein biosynthesis. Importantly, ex-translational functions appear only when tRNA is uncharged. The most spectacular examples come from bacterial cells and include induction of the stringent control, regulation of transcription of some operons, and control of replication of ColE1-type plasmids (Węgrzyn & Węgrzyn, 2008). In eukaryotes, it has been observed that cells can respond to starvation conditions in a tRNA-dependent manner. In yeast and mammals, tRNA induces phosphorylation of eukaryotic initiation factor 2 through binding to the Gcn2 kinase which modulates the transcription of amino acid biosynthesis genes and reduces total protein biosynthesis (Dong *et al*., 2000; Hao *et al*., 2005). Moreover, during stress responses, tRNA transcription is down-regulated, and there is a retrograde transport of tRNAs into the nucleus (Hopper & Shaheen, 2008). Recent studies have described also a novel aspect of stress responses wherein cytosolic tRNAs are processed to shorter forms. For many years tRNA fragments were thought to be by-products of tRNA metabolism with no biological function. In recent years, however, many research groups have revealed that tRNA cleavage products can have multiple biological functions eg. during gene expression regulation, tumour progression or cell proliferation (see the next paragraph).

tRNA-DERIVED FRAGMENTS

tRNA-derived fragments are present in all three domains of life and their amount is often increased during non-physiological conditions. Several sequencing projects have demonstrated the presence of tRNA-derived fragments in various eukaryotic organisms, including the fruit fly *Drosophila* (Aaravin *et al*., 2003), the fungus *Aspergillus* (Jöchl *et al.,* 2008), yeast *S. cerevisiae* (Zywicki *et al*., 2012), human cell lines (Kawaji *et al*., 2008; Haussecker *et al*., 2010) and pumpkin phloem sap (Zhang *et al*., 2009).

Since their discovery, the terminology regarding small RNAs derived from tRNAs has been highly inconsistent. Such names as tRNA halves, tRNA-derived RNA fragments (tRFs) (Lee *et al*., 2009), stress-induced small RNAs (tiRNAs) (Yamasaki *et al*., 2009), tRNA-derived small RNAs (tsRNAs) (Haussecker *et al*., 2010) or urinary bladder carcinoma RNAs (ubcRNAs) (Zhao *et al*., 1999) are used by various research groups and refer to similar entities. Recently, a nomenclature based on tRNA fragment size and the part of the tRNA molecule from which fragments are derived was proposed (Sobala & Hutvagner, 2011). According to this, tRNA fragments can be divided in two major classes: tRNA halves and small tRNA fragments (tRFs).

tRNA halves have a size of 30–35 nt and are produced by a cleavage in the anticodon loop. The enzyme responsible for this cleavage in higher eukaryotes in response to stress conditions is angiogenin, a member of the RNase A family (Yamasaki *et al.,* 2009). Under normal conditions, angiogenin is translocated into the nucleus, but is released into the cytoplasm under certain stress conditions. However, cleavage by angiogenin can be regulated by tRNA methylation mediated by the DNA methyltransferase Dnmt237, or by the ribonuclease inhibitor RNH1 (Nawrot *et al*., 2011). Moreover, angiogenin is a potent vascularization agent in normal and malignant cells and a potential oncogene (Zhao *et al*., 1999).

Small tRFs of approximately 20 nt in length are derived from either the 5’ or 3’-end of mature tRNAs (5’tRF and 3’tRFs). tRFs are also produced from 3’-pre-tRNA trailers (3’U tRFs) during processing of pre-tRNAs by RNase Z. The mechanisms by which 5’- and 3’tRFs are generated in the cell are not yet completely understood but it has been proposed that Dicer may be involved in tRFs production, despite the fact that tRNA does not meet the structural criteria of a classical Dicer substrate. Human tRNA fragments have been identified in pools of small RNAs co-purifying with Argonaute and Piwi complexes, suggesting that such tRNA fragments could function as siRNAs or miRNAs (Haussecker *et al.,* 2010; Kawamura *et al*., 2008; Lau *et al*., 2006). Haussecker and coworkers investigated the propensity of 3’tRFs (both types) to associate with Argonaute proteins and their effect on luciferase reporter genes (Haussecker *et al.,* 2010). They found that both types of 3’tRF associated with Argonaute proteins, but often more effectively with Ago3 and Ago4 than Ago1 or Ago2. They found that 3’tRFs had a moderate effect on reporter transgene silencing, but 3’U tRFs did not. However, upon co-transfection of a small RNA complementary to 3’U tRF, they found that tRF preferentially associated with Ago2 and silenced a reporter transgene, a phenomenon they termed sense-induced transgene silencing (SITS). This is in contrast with results normally obtained in the miRNA field where sequences complementary to miRNAs relieve repression. Haussecker and coworkers suggested that the double-stranded perfect RNA helix produced when a sense strand is present causes the more efficient loading into Ago2, which is consistent with *in vitro* studies showing that Ago2’s slicer activity causes more efficient loading of perfect duplexes (Yoda *et al*., 2010). Serendipitously, Lee and coworkers (2009) and Haussecker and coworkers (2010) chose to characterize the same tRF, called cand45 by Haussecker and tRF-1001 by Lee. Whereas Lee and coworkers (2009) did not look at Argonaute association of tRF-1001, they found out that its knockdown by siRNA decreased cell proliferation and re-addition increased proliferation. Hence, Lee and coworkers (2009) found a function for 3’U tRFs in a situation where Haussecker and coworkers (2010) showed them to be primarily associated with Ago3 and Ago4.

Cleavage of tRNAs in specific position has several potential consequences. For instance, tRNA cleavage could inhibit translation by depletion of tRNA pool. However, full-length tRNA is not significantly depleted in any of the studies published to date, regardless of tRNA fragment levels. Notably, stress-induced cleavage of tRNAs is not a mechanism to degrade misprocessed or hypomodified tRNAs. There are some indirect evidences suggesting that cleaved tRNAs themselves may function to inhibit translation: (i) stable processing products (5’tRFs as well as 3’tRFs) derived from all nuclear-encoded tRNAs have been co-purified with yeast ribosomes (Zywicki *et al*., 2012); (ii) *Curbita maxima* phloem-derived

RNA pool, which contains among others tRNA fragments, have a potential to inhibit the overall protein biosynthesis in wheat germ extract (Zhang *et al*., 2009); (iii) transfection of endogenous 5'tRFs, but not 3'tRFs into mammalian U2OS cells results in a global translation inhibition (Yamasaki *et al*., 2009).

The function of 5'tRFs in human cells has been recently elucidated by Sobala and Hutvagner (Sobala & Hutvagner, 2013). The data showed the potential of 5'tRFs to inhibit the translation of reporter genes *in vitro* and *in vivo*, an effect that does not require any complementary target sites in the reporter sequence, but does require a universally conserved "GG" dinucleotide in the tRF. This first result opened the possibility that 5'tRFs, like other tRNA-derived fragments, are involved in regulating gene expression. These data show notable parallels to a recent study finding that tRNA fragments have a direct inhibitory effect on protein translation in the archeon *Haloferax volcanii* (Gebetsberger *et al*., 2012). In that study, Gebetsberger and coworkers show that 26 nt 5'tRFs are able to inhibit translation. Specifically, tRF(Val) is able to inhibit peptide bond formation, whereas in contrary less abundant tRF(Ile) is not. The fact that 5'tRFs are able to inhibit translation across domains of life suggests that this may be the old way by which organisms control protein translation. Interestingly, a recent study by Ivanov *et al.* suggested that specific endogenous 5'tRFs inhibit translation initiation in oxidatively stressed human cell lines by recruiting eIF4E/G/A from capped mRNAs or eIF4G/A from uncapped mRNAs (Ivanov *et al*., 2011).

tRNA FRAGMENTS AND CANCER

tRNA fragments have been observed in the urine and sera of cancer patients, with levels correlated with the tumour burden already in 1970's (Borek *et al*., 1977; Speer *et al*., 1979; Walkers *et al*., 1975). It was then already shown that there is a very high turnover of tRNA in tumor tissue. In 27 cancer patients, 26 of them had elevated levels of one or more of seven tested breakdown products of tRNA in urine. It was also estimated that the level of two of these markers is connected with the stage of cancer and correlates with cancer progression.

Also, modern techniques have recently shown that tRNA is processed to shorter forms in cancer. In 2007 Lui and coworkers presented the results of deep-sequencing method and characterized small RNA profiles for six human cervical carcinoma cell lines (Lui *et al.,* 2007). Out of more than 7000 small RNA clones, 8% of them represented tRNA fragments.

In human prostate cancer the most abundant group of small RNA, just after miRNAs, are tRFs (Lee *et al*., 2009). Lee and coworkers (2009) reported a deep-sequencing analysis of total small RNAs from two cancer cell lines. They got >600 000 reads that included 17 RNAs, 18–22 nt in length, that aligned with transfer RNA sequences. Five of them were derived from the 5' ends of mature tRNAs, eight were derived from the 3' ends of mature tRNAs, and four were derived from the 3' trailer regions of pre-tRNAs. Concerning biological function of tRFs, Lee and coworkers found out that tRF-1001, derived from the 3' end of a Ser(TGA) tRNA precursor, is required for cell proliferation and is highly expressed in a wide range of cancer cell lines of many different lineages. After knocking down this specific tRF, dramatic loss in cell proliferation and their viability was observed. This effect was blocked by the co-introduction of a 2'-*O*-methyl version of tRF-1001 together with siRNA. What is more, DNA synthesis was decreased and high levels of cells in G2 phase occurred. Another relation of tRF(Ser) to the cancer is the origin of this tRNA fragment – it is generated by the cytoplasmatic endoribonuclease ELAC2 (a homolog of RNase Z), which is a prostate cancer susceptibility gene (Tavtigian *et al.,* 2001).

Interestingly, in human cells, tRFs may act as microRNA-like molecules and as post-transcriptional regulators. Possible endogenous targets of miRNA-like-tRFs have been demonstrated by Li and coworkers in 2012. Using sequencing, computational analysis and northern blot assays, they showed high abundance of 3' and 5' tRNA terminal fragments in B lymphoma BCP1 cell line (Li *et al*., 2012). They have shown that 3'tRFs but not the 5' tRFs are highly complementary to human endogenous retroviral sequences in the genome. Despite their independence from Dicer processing, these tRFs associated with Ago2 and were capable of down-regulating target genes by transcript cleavage *in vitro*. They have tested an artificial target mRNA (100 nt) containing a region (17–18 nt) fully complementary to the endogenous 3' tRFs from the two different tRNAs: Leu(CAG) and His(GUG). As a result, endogenous 3' tRFs directed Ago2-mediated cleavage.

Research performed recently by Maute and co-workers showed also that tRNA 3' fragment is able to modulate proliferation and DNA damage response in human B–cell lymphoma (Maute *et al.,* 2013). Moreover, this tRNA fragment has a functional characteristics of microRNAs, namely it undergoes DICER1-dependent cleavage, it binds to all human Argonaute proteins and regulates gene expression at post-transcriptional level in sequence-dependent manner, such as miRNAs. It has been shown that this miRNA-like-tRF is capable of repressing a set of endogenous genes, including *RPA1* gene, which plays an important role in several cellular processes such as replication, DNA repair and recombination. Interestingly, this tRF is down-regulated in lymphoma cell lines. As a result, an increase in RPA1 protein production was observed, and the malignant cells had a tendency for increased growth.

tRNA and its fragments may be also involved in the modulation of cell growth (Zhao *et al.,* 1999). tRNA fragments purified from human urinary bladder carcinoma cells and added to the medium could inhibit endothelial cell proliferation, but not other cell lines such as human bladder carcinoma cells and bovine smooth muscle. This indicates that tRFs may take part in a physiologically relevant regulatory system in the extracellular fluids of cancer cells.

Recent research performed by Martens-Uzunova revealed that the tRNA fragments could be present in high amounts in metastatic samples. The expression of the entire small transcriptome was examined in prostate cancer cells using Illumina/Solexa deep sequencing. Interestingly, it was shown that most tRNA-derived fragments in prostate cancer library had a size of ~18 nt, while in the lymph node prostate cancer library tRF had size range of 27 nt indicating different processing of tRNA (Martens-Uzunova *et al.,* 2012).

tRNAs AND CANCER

In cancer cells, there has been observed a correlation between an increased growth rate and proliferation, metabolism and protein synthesis (Gillies *et al*., 2008; Jones

& Thompson, 2009; Cairns *et al.*, 2011; White, 2005; Mei *et al.,* 2010). Studies based on comparative expressed sequence hybridization determined that there is aberrantly high level of rRNA and pre-rRNA in every sample of the collection of examined tumours (Williamson *et al.,* 2006). There is also some evidence showing that tRNA expression in cancer-derived cells is higher than in normal tissues, which may be one of the causes of that phenomenon (Pavon-Eternod *et al.*, 2009; Mei *et al.,* 2010; Mahlab *et al.,* 2005; Zhou *et al.,* 2009). This over-expression is often associated with overexpression of TFIII factor components (White, 2005). Based on a disparate collection of tumours examined and number of publications confirming that fact, one can assume that overexpression of tRNA might be the general feature of cancer.

In 2009, Pavon-Eternod and coworkers used tRNA microarrays for measuring genome-wide tissue-specific expression levels of tRNA molecules in three non-cancer derived breast epithelial cell lines and in six breast cancer cell lines. They have shown that in the breast cancer lines there is a global overexpression of all tRNA species. Specifically, in cancer-derived *versus* non-cancer-derived cell lines, all nuclear-encoded tRNA expression increased up to 3-fold and mitochondrial-encoded tRNA expression increased up to 5-fold. However, this increased level of tRNA did not have an influence on tRNA composition in cancer tissue. Moreover, Mahlab and coworkers in 2012 showed that the composition of tRNA isoacceptors in healthy, transformed and cancerous cells remains identical. However, it was proved already in the 80's that tRNA from cancerous tissues have different post-translational modification pattern than tRNA from healthy tissues (Solomon *et al.*, 1985; Kuchino *et al.,* 1982; Borek *et al.*, 1987). Moreover, not only tRNAs are over-expressed in cancer tissue. Other products of RNA polymerase III are also over-expressed (Kuchino & Borek, 1978).

Recent data also show that deregulation of tRNA expression can have a strong impact on cancer cell translation process. Pavon-Eternod and coworkers in 2013 overexpressed initiator $tRNA^{Met}$ in MCF10 and 184A1 epithelial cell lines. This process has changed the levels of other tRNAs in both epithelial cell lines and caused reprograming of the global tRNA expression profile (Pavon-Eternod *et al.*, 2013). Surprisingly, there is a very poor correlation between tRNA levels in breast cancer cell lines and the tRNA levels induced by $tRNA^{Met}$ overexpression. While tRNA carrying charged and polar amino acids were present in most tRNA-overexpressing breast cancer cell lines, there was no such trend observed in $tRNA^{Met}$-overexpressing cells. Another interesting observation in this phenomenon was an increase of cell metabolic activity and cell proliferation speed in $tRNA^{Met}$-overexpressing cells. Authors were very cautious in interpreting these results, however, they clearly showed how limited is our knowledge about the regulation of individual tRNA expression in cells. Moreover, cancer cells with higher level of tRNA require higher level of amino acids for the charging process. In different study, Zhou and coworkers in 2009 proved that bortezomid, the proteasome inhibitor, approved for the multiple myeloma treatment by FDA in 2003, slows down the overall metabolism in multiple myeloma cells by reducing the amino acids recycling (Zhou *et al.*, 2009). Thus, bortezomib treatment results in decreased aminoacylation level of tRNAs, which is required for cancer cells protein biosynthesis.

One more evidence for correlation between high levels of tRNA and cancer was published by Mei and coworkers in 2010. The results showed that both mitochondrial and cytosolic tRNAs (although to lesser extend) bind to the cytochrome c and, therefore, inhibit the activation of caspase 9, thus preventing apoptosome formation. Moreover, microinjection of tRNA inhibits also cytochrome *c*-induced apoptosis. This results show that tRNA may be one of the most important factors preventing cancerous cells from apoptosis.

Following previous data we can assume that tRNA plays an outstanding role in cancer genesis and biology. The question is how can we use this knowledge for the cancer treatment? Zhou and coworkers in 2012 published their results on an interesting new form of therapy which utilizes "killer tRNA" (Zhou *et al.*, 2012). This "killer tRNA" is an engineered human serine tRNA with anticodon which recognizes isoleucine anticodon — $tRNA^{Ser}$(AUU). The introduction of such $tRNA^{Ser}$(AUU) into the cells leads to many serine to isoleucine substitutions during mRNA translation, which interrupts protein patterns and leads to apoptosis. These authors injected $tRNA^{Ser}$(AUU) to breast tumor induced in mice and noticed that tumor formation and growth were completely inhibited. Mischarged tRNAs do not often occur in normal cells, it is estimated that only 10^3–10^4 of tRNA molecules are charged with non-cognate amino-acid, which indicates high accuracy of amino-acylation reaction (Loftfield & Vanderjagt, 1972). However, Lee and coworkers in 2006 discovered that a defect in a single tRNA synthetase in mouse leads to the production of heterogeneous misfolded proteins and to neurodegeneration, caused by mischarged tRNA. These results provide a novel mechanism for the generation of misfolded proteins, which are associated with human diseases, but also reveal potential risk of the "killer tRNA" therapy. Moreover, this therapy shares also the same problem with other RNA based therapies — delivering this tRNA to specific cells of interest. The killer $tRNA^{Ser}$(AUU) is proven to kill preferentially the tumor cells than the normal epithelial cells. However, the possibility of toxicity to normal cells makes the developing of targeted delivery to tumor cells a high priority required to develop the therapeutic potential of "killer tRNA".

CONCLUSIONS

Recent identification of new classes of ncRNAs implicated in important steps of cancer formation and progression reinforces the role of these transcripts in the process of tumor genesis. While the majority of investigations have been focused on miRNAs, it is now clear that other non-coding RNAs (like tRFs) may function to direct genetic programming, thus contributing to cancer initiation and/or progression. Moreover, some of the newly identified ncRNAs can be used now as the biomarkers and also as the therapy targets. Thus, an additional challenge for cancer biologists in the years ahead will be the identification of all the defects associated with both protein-coding and non-coding genes in tumour cells. This in turn will lead to an improved understanding of the molecular underpinnings of cancer and eventually to the development of the novel biomarkers and targeted therapies in cancer.

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