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DEREGULATED EXPRESSIONS OF *MYC* ALTER THE EXPRESSIONS OF tRNAS IN BREAST CANCER CELLS

MYC MEME KANSERİ HÜCRELERİNDE tRNA'LARIN İFADESİNİ REGÜLE EDER

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ABSTRACT

Objective: The protein synthesis process is started with DNA being transcribed into mRNA in the nucleus, then mRNA is transported to the cytoplasm and attaches to a ribosome in order to be translated into a protein. Protein synthesis not only occurs in the cytoplasm. Mitochondria by itself also has its own genetic system. Therefore mtDNA can be replicated, transcribed, and translated. *MYC* oncogene drives the generation of many cancer types. *MYC* also controls some target genes related to ribosome biogenesis and protein synthesis. Increased expressions of tRNA synthetases have been found to be important players in promoting tumor growth in various types of human cancers. In this study we aimed to identify deregulated expressions of tRNAs that are players both in the nucleus and mitochondria in a MYC-dependent manner in breast cancer cells.
Material and Methods: Cells were infected with lentiviral MYC shRNA/overexpression vectors in order to manipulate MYC expressions. TRIZOL reagent was used for RNA isolation and libraries were generated for sequencing. The quality of the RNA samples and libraries was assessed using Agilent BioAnalyzer. Sequencing was performed on the Illumina HT2500 platform.
Results: We obtained 6 nuclear-encoded mitochondrial tRNAs and 65 nuclear tRNAs as a result of deregulated expression of *MYC*.
Conclusion: This study reveals *MYC-dependent* regulation of tRNAs in breast cancer. Further functional studies are required for underlying molecular mechanisms.

Keywords: tRNA, next-generation sequencing, breast cancer

ÖZ

Amaç: Protein sentezi süreci, DNA'nın çekirdekte mRNA'ya kopyalanmasıyla başlar, ardından mRNA, bir proteine çevrilmek üzere sitoplazmaya taşınır ve ribozoma bağlanır. Protein sentezi sadece sitoplazmada gerçekleşmez. Mitokondri de kendi genetik sistemine sahiptir. Bu nedenle mtDNA'da replikasyon, transkripsiyon ve translasyon gerçekleşir. MYC onkogeni, birçok kanser türünün gelişimine neden olur. MYC ayrıca ribozom biyogenezi ve protein sentezi ile ilgili bazı hedef genleri kontrol eder. tRNA sentetazlarının artan ifadelerinin, çeşitli insan kanseri türlerinde tümör büyümesini desteklemede önemli rolü olduğu bulunmuştur. Bu çalışmada, nükleus ve mitokondride görev yapan tRNA'ların *MYC*'e bağımlı ifadelerini belirlemeyi amaçladık.
Gereç ve Yöntem: Hücreler lentiviral *MYC* overekspresyon/shRNA vektörleri ile infekte edildi. RNA izolasyonu için TRIZOL reaktifi kullanıldı ve yeni nesil dizileme için kütüphaneler hazırlandı. RNA örneklerinin ve kütüphanelerin kalitesi, Agilent BioAnalyzer kullanılarak değerlendirildi. Dizileme, Illumina HT2500 platformunda yapıldı.
Bulgular: Meme kanseri hücrelerinde *MYC* ifade değişimine bağlı olarak 65 nuklear tRNA ve 6 nükleer kodlanmış mitokondriyal tRNA saptadık.
Sonuç: Bu çalışma, meme kanserinde tRNA'ların *MYC*'e bağlı regülasyonunu ortaya koymaktadır. Moleküler mekanizmaların aydınlatılması için daha ileri fonksiyonel çalışmalar gereklidir.

Anahtar Kelimeler: tRNA, yeni nesil dizileme, meme kanseri

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INTRODUCTION

The protein synthesis process is started with DNA being transcribed into mRNA in the nucleus, then mRNA is transported to the cytoplasm and attaches to a ribosome in order to be translated into a protein. In this journey, the ribosome reads the chain of codons in the mRNA strand and tRNA translates each three-base codon on the mRNA into an amino acid which creates a polypeptide, that can be processed into a protein. Transfer RNA is an important player in protein synthesis and besides, they also have important roles in the epigenetic regulation of gene expression in various cancers (1).

Protein synthesis not only occurs in the cytoplasm. Mitochondria by itself also has its own genetic system. Therefore mtDNA can be replicated, transcribed, and translated. The double-stranded and circular mitochondrial genome is within a size of 16.5 kb and contains 37 genes encoding 13 proteins, 2 rRNAs and 22 tRNAs (2). The localization of tRNAs are on both the heavy and light strands of the mitochondrial genome and they are transcribed as long polycistronic precursors (3). The tRNA punctuation model was described in 1981 and this model reveals that coding sequences are generally separated by few nucleotides and long polycistronic precursors are processed into mature mRNA and rRNA by cleavage of the 5’ and 3’-ends of the flanking tRNAs. (4). tRNAs participate in the protein translation process that mRNAs are converted into an amino acid chain. In mitochondrial translation, the primary processing occurs through cleavage at the 5’ end of tRNA by mitochondrial RNase P4 and 3’ end by the mitochondrial RNase Z (5,6). mt-RNase P is composed of mitochondrial RNase P proteins 1,2,3 and MRPP1-3 (7). There are four steps of mitochondrial protein translation including initiation, elongation, termination, and ribosome recycling. Mitochondrial translation mechanisms are much more similar to prokaryotic translation (8). For the mitochondrial protein synthesis, MTIF2, MTIF3 (initiation factors), EFTU, EF-TS, mtEF-G1 (elongation factors), MTRF1L (a release factor), MRRF and EF-G2mt (ribosome recycling factors) are required. A deficiency of protein translation factors, mt-tRNAs, mitochondrial ribosome proteins, etc results in abnormal mitochondrial translation, which is associated with a variety of diseases, such as cancer, nervous system diseases, and cardiovascular diseases (9-13).

The structural changes of mtDNA in encoded protein subunits unshared electron transport function and ROS production increases. Carcinogenesis occurs depending on enhanced oxidative stress related to an increased ROS production in mitochondria (14). Increased expressions of tRNA synthetases have been found to be important players in promoting tumor growth in various types of human cancers (15,16). Many studies suggested the important roles of tRNAs in cancer as potential biomarkers.

MYC oncogene drives the generation of many cancer types including breast cancer (17-19). *MYC* amplification and overexpression in breast cancer are related to poor outcomes for the patients (20). *MYC* also controls some target genes that have roles in ribosome biogenesis and protein synthesis (21). After *MYC* binding sites are identified, transcriptional targets are explored and 400 nuclear genes encoding mitochondrial proteins were found to be targets of *MYC* by chromosomal immunoprecipitation analysis of promoter microarrays including genes related to protein import, mitochondrial ribosome, mitochondrial transcription/translation and complex assembly, whereas 198 genes were induced as a result of ectopic expression of *MYC* (22,23). Besides, *MYC* was found to regulate mitochondrial gene expression indirectly via microRNAs (24).

It has been shown that the regulation of tRNA ligases by MYC is imputed as a fundamental contributor to *MYC*-driven cell growth in Drosophila (25). Even small RNA sequencing performs successful mapping and alignment of gene transcripts and small RNA types including miRNAs, snRNAs, rRNA, and tRNAs, most workflows have been developed for miRNA analysis. But pipelines for the analysis of transfer RNAs are rare. Besides, the roles of tRNAs on cancer development are not very well known. Both mRNAs and tRNAs are important in protein synthesis. Therefore, in the direction of the knowledge that *MYC* regulates both nuclear and mitochondrial genes, here we aimed to illustrate in breast cancer cells if deregulated expressions of MYC also affect the expression levels of tRNAs that are players both in the nucleus and mitochondria.

MATERIALS and METHODS

Cell culture

MDA-MB-231 cells are cultured in RPMI1640 Medium (GIBCO, USA) containing heat-inactivated 5% FBS (GIBCO, USA), 1% Penicillin-Streptomycin (GIBCO, USA) and 1% L-glutamine (GIBCO, USA) and MCF-7 cells are cultured in DMEM Medium (SIGMA, USA) containing heat-inactivated 5% FBS (GIBCO, USA), 1% Penicillin-Streptomycin (GIBCO, USA) and 1% L-glutamine (GIBCO, USA) and are incubated at 37°C, with 5% CO2 and 95% humidity in air.

Lentivirus infection

Inducible lentiviral overexpression/shRNA vectors were designed by Dr.Onur Tokgun (26). MDA-MB-231 and MCF-7 cells were counted and seeded with 3ml medium. Twenty four hours after, cells were infected with a combination of 20 µl lentivirus and 8 µl Polybrene (Sigma Aldrich). After removing the cell media, cells were washed with PBS (GIBCO) twice and replaced with the RPMI140 medium supplemented with 10% Tet-Free FBS (Clonetech) 24 hours after infection. Cells were selected with puromycine in a concentration of 2 µg/ml and induced with Doxycycline (DOX) in a concentration of 2.0 µg/ml.

RNA isolation

Total RNA was isolated from MCF-7 DX-/DX+, MDA-MB-231 DX-/DX+ cells in a density of $5x10^7$ cells grown on T175 flasks. Cell pellets were lysed in 500 µl TRIzol reagent. Subsequently, 100 µl of chloroform was used for phase separation, 100% isopropanol for RNA precipitation and ice-cold 75% ethanol for washing. RNA was eluted in 30 µl RNase-free water. The RNA integrity and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

qRT-PCR

Total RNA was reverse transcribed using a High-capacity cDNA reverse transcription kit (Thermo Scientific, Waltham, MA, USA). qRT-PCR reactions were prepared using BioRAD iTaq Universal SYBR Green Master mix. Oligonucleotide sequences are as follows: MYC, F: CTTCTCTCCGTCCTCGGATTCT R: GAAGGTGATCCAGACTCTGACCTT; GAPDH, F: AGGTCGGTGTGAACGGATTTG R: GGGGTCGTTGATGGCAACA. GAPDH was used as an endogenous control. mRNA expression of Myc between DX+ versus DX− was measured using Eq. $2^{-\Delta\Delta Ct}$.

Small RNA sequencing

Small RNA sequencing was performed at the Macrogen company (South Korea) using the Illumina HT2500 platform. The RNA yield and size distribution of the libraries were analyzed with an Agilent 2100 Bioanalyzer. Libraries generated from 50 ng/µL total RNA samples using the SMART-seq v4 (Takara, America). First, poly(A) tail is added to input RNA using Poly(A) Polymerase in order to facilitate oligo(dT)-primed cDNA synthesis. Then adapter-linked RNA fragments are transformed into cDNA fragments and followed by PCR purification. The sizes of the amplified cDNA fragments were checked using a Bioanalyzer DNA High sensitivity chip. The cDNA fragments are sequenced according to the read length using the "sequence by synthesis" method on the Illumina platform.

Bioinformatic analysis

Raw sequence readings were filtered and the adapter sequences were removed from the raw sequence readings using the CutAdapt program. The clipped reads are clustered for matching 100% of the sequence and read length. Clustered reads were then aligned with the reference genome (hg19). To classify sRNA types, clustered reads were mapped to the reference genome and non-coding RNA database Rfam v9.1. The expression levels of RNA transcripts in high throughput data are calculated as FPKM (fragments per kilobase of transcript per million reads mapped) (27).

Statistical analysis

The graphs, calculations, and statistical analyses were performed using GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA, USA). Unpaired Student's t-test was used for comparisons of differential expressions of *MYC*. Statistical results with ****p <0.0001 were considered as statistically significant.

RESULTS

The deregulated expression levels of *MYC* in breast cancer cells are shown in Figure 1. The total base number, number of readings, GC percentage, Q20 (%), and Q30 (%) were calculated and 3' adapter sequences were removed using the "Cutadapt" program. Sequences smaller than 15 nucleotides were filtered and the reads were matched to the reference genome (hg19), respectively (Table 1). The length analysis of sRNA population was categorized by performing a length analysis.

Rfam v9.1 and Genbank databases were used to determine sRNAs as well as tRNAs. When sRNA sequences are paired with RNAs from Genbank and Rfam databases, tRNAs (MCF-7 DX- 2.71%, MCF-7 DX+ 1.53%, MDA-MB-231 DX- 1% and MDA-MB-231 DX+ 1.66%) was one of the most common sRNA classes in cells. In total, 71 tRNAs were determined, of which 6 of them were nuclear-encoded mitochondrial tRNAs (Table 2). A HeatMap analysis of detected tRNAs are shown in Figure 2.

Table 1: Raw Data Statistics and Filtered Number of Reads

Sample	Total Reads	GC (%)	Q20 (%)	Q30 (%)
MDA-MB-231 DX-	26.377.797	43.03	84.74	79.81
MDA-MB-231 DX+	32.880.597	42.28	82.79	77.62
MCF-7 DX-	34.892.493	39.42	86.16	81.82
MCF-7 DX+	45.257.303	36.98	92.47	89.11
Filtered short reads of less than 15 bases				
MDA-MB-231 DX-	12.245.859	68.76	97.03	93.51
MDA-MB-231 DX+	14.806.966	74.96	97.67	94.72
MCF-7 DX-	13.832.085	78.66	97.42	94.33
MCF-7 DX+	14.696.527	63.74	97.79	95.08

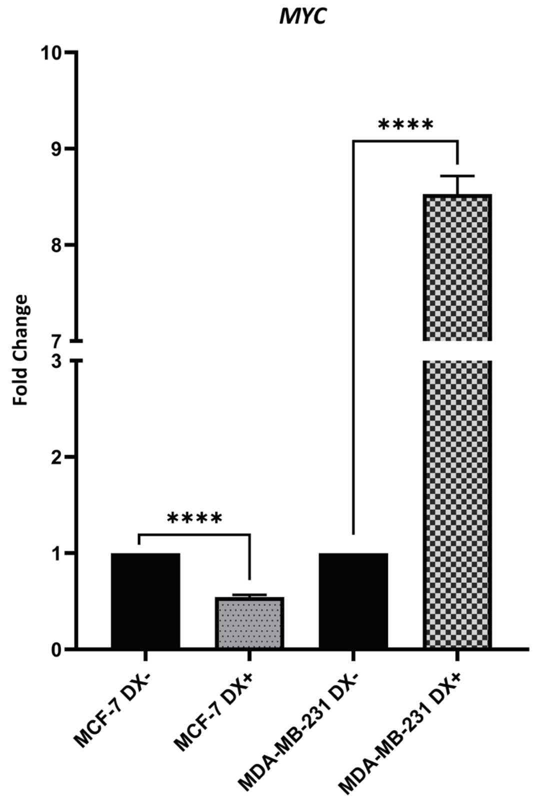

Figure 1: Expression levels of *MYC* after *MYC* inhibition/overexpression in breast cancer cells by lentiviral vectors (**** p<0.0001).

Table 2: Deregulated expressions of tRNAs depending on *MYC* manipulation

Gene_Name	tRNA_Type	Position	MDA-MB-231 DX-	MDA-MB-231 DX+	MCF-7 DX-	MCF-7 DX+
nmt-tRNA-Gln-CTG-1-1	Gln-CTG	chr10:20036618-20036674:-	0	3	319	7
nmt-tRNA-Gln-TTG-7-1	Gln-TTG	chr2:131029911-131029982:-	81	113	2964	542
nmt-tRNA-Gln-TTG-9-1	Gln-TTG	chr2:132143133-132143204:+	86	131	3206	628
nmt-tRNA-Leu-TAA-4-1	Leu-TAA	chrX:55207755-55207829:-	1498	325	91	171
nmt-tRNA-Ser-TGA-1-1	Ser-TGA	chr2:131033024-131033093:-	73	121	957	240
nmt-tRNA-Ser-TGA-3-1	Ser-TGA	chr17:22028179-22028247:-	16	56	743	108
tRNA-Ala-AGC-10-1	Ala-AGC	chr6:26687485-26687557:+	1	48	20	3
tRNA-Ala-AGC-4-1	Ala-AGC	chr6:28626014-28626085:-	1	411	98	32
tRNA-Ala-CGC-1-1	Ala-CGC	chr6:26553731-26553802:+	1	406	98	22
tRNA-Ala-CGC-2-1	Ala-CGC	chr6:28641613-28641684:-	1	393	61	23
tRNA-Ala-CGC-3-1	Ala-CGC	chr2:157257281-157257352:+	1	425	72	56
tRNA-Ala-TGC-3-2	Ala-TGC	chr12:125406301-125406372:-	1	392	72	23
tRNA-Ala-TGC-4-1	Ala-TGC	chr12:125424512-125424583:+	1	407	115	65
tRNA-Arg-CCG-1-1	Arg-CCG	chr6:28710729-28710801:-	1	32	121	49
tRNA-Arg-CCG-2-1	Arg-CCG	chr17:66016013-66016085:-	1	882	468	177
tRNA-Arg-CCT-1-1	Arg-CCT	chr17:73030001-73030073:+	1	372	85	38
tRNA-Arg-CCT-4-1	Arg-CCT	chr7:139025446-139025518:+	1	380	249	38
tRNA-Arg-TCG-3-1	Arg-TCG	chr17:73031208-73031280:+	1	31	125	53
tRNA-Arg-TCT-5-1	Arg-TCT	chr6:27529963-27530049:+	1	28	151	33
tRNA-Asp-GTC-1-1	Asp-GTC	chr12:98897281-98897352:+	34	1098	2804	1508
tRNA-Asp-GTC-2-1	Asp-GTC	chr1:161410615-161410686:-	83	1314	3090	2264
tRNA-Asp-GTC-3-1	Asp-GTC	chr6:27551236-27551307:-	83	981	3021	1462
tRNA-Asp-GTC-4-1	Asp-GTC	chr9:77517990-77518061:-	81	947	2730	1356
tRNA-Glu-CTC-1-1	Glu-CTC	chr1:145399233-145399304:-	8	1797	2363	1263
tRNA-Glu-CTC-3-1	Glu-CTC	chr13:42030061-42030132:-	1	577	223	163
tRNA-Glu-TTC-1-1	Glu-TTC	chr2:131094701-131094772:-	6	346	810	240
tRNA-Glu-TTC-2-1	Glu-TTC	chr13:45492062-45492133:-	7	363	873	278
tRNA-Glu-TTC-3-1	Glu-TTC	chr1:17199078-17199149:+	7	1243	2063	684
tRNA-Glu-TTC-4-1	Glu-TTC	chr1:16861774-16861845:-	7	1272	2179	710
tRNA-Gly-CCC-1-1	Gly-CCC	chr1:16872434-16872504:-	53	449	200	133
tRNA-Gly-CCC-5-1	Gly-CCC	chr1:17053780-17053850:+	6	309	109	64
tRNA-Gly-CCC-6-1	Gly-CCC	chr1:149680215-149680275:-	53	380	161	98
tRNA-Gly-GCC-1-1	Gly-GCC	chr1:161413094-161413164:+	59	613	1028	270
tRNA-Gly-GCC-2-1	Gly-GCC	chr1:161493637-161493707:-	59	625	1058	270
tRNA-His-GTG-1-1	His-GTG	chr1:145396881-145396952:-	1	101	226	300
tRNA-Ile-AAT-1-1	Ile-AAT	chr6:58149254-58149327:+	1	116	787	115
tRNA-Ile-AAT-2-1	Ile-AAT	chr6:27655967-27656040:+	1	65	385	57
tRNA-Ile-AAT-5-1	Ile-AAT	chr6:26554350-26554423:+	1	118	787	115
tRNA-Ile-AAT-6-1	Ile-AAT	chr6:26745255-26745328:-	1	65	384	57
tRNA-Ile-AAT-9-1	Ile-AAT	chr6:27241739-27241812:+	1	31	336	12
tRNA-Leu-AAG-1-1	Leu-AAG	chr5:180524474-180524555:-	1	196	113	33
tRNA-Leu-AAG-2-1	Leu-AAG	chr5:180614701-180614782:+	1	187	137	36

tRNA-Leu-AAG-3-1	Leu-AAG	chr6:28956779-28956860:+	1	237	122	36
tRNA-Leu-AAG-4-1	Leu-AAG	chr6:28446400-28446481:-	1	156	108	25
tRNA-Leu-CAA-1-2	Leu-CAA	chr6:28908830-28908934:+	1	129	43	9
tRNA-Leu-CAA-2-1	Leu-CAA	chr6:27573417-27573524:-	1	154	34	8
tRNA-Leu-CAA-3-1	Leu-CAA	chr6:27570348-27570454:-	1	178	36	9
tRNA-Leu-CAA-4-1	Leu-CAA	chr1:249168054-249168159:+	1	158	34	8
tRNA-Leu-TAG-1-1	Leu-TAG	chr17:8023632-8023713:-	1	197	113	33
tRNA-Leu-TAG-2-1	Leu-TAG	chr14:21093529-21093610:+	1	185	135	34
tRNA-Lys-CTT-1-1	Lys-CTT	chr14:58706613-58706685:-	2	76	501	34
tRNA-Lys-CTT-2-1	Lys-CTT	chr1:145395522-145395594:-	1	92	496	35
tRNA-Lys-CTT-3-1	Lys-CTT	chr16:3207406-3207478:-	1	69	484	30
tRNA-Lys-CTT-4-1	Lys-CTT	chr16:3241501-3241573:+	3	71	507	37
tRNA-Lys-CTT-5-1	Lys-CTT	chr16:3230555-3230627:-	1	57	423	22
tRNA-Lys-CTT-chr7-30	Lys-CTT	chr7:96770888-96770952:-	1	41	402	20
tRNA-Lys-TTT-1-1	Lys-TTT	chr16:73512216-73512288:-	1	123	156	19
tRNA-Lys-TTT-2-1	Lys-TTT	chr11:122430655-122430727:+	1	126	163	21
tRNA-Lys-TTT-3-1	Lys-TTT	chr1:204475655-204475727:+	1	123	91	19
tRNA-Lys-TTT-5-1	Lys-TTT	chr11:59323902-59323974:+	1	107	85	14
tRNA-Lys-TTT-6-1	Lys-TTT	chr6:27302769-27302841:-	1	107	59	11
tRNA-Met-CAT-1-1	Met-CAT	chr8:124169470-124169542:-	1	64	498	105
tRNA-Met-CAT-6-1	Met-CAT	chr16:87417628-87417700:-	1	52	231	78
tRNA-Pro-AGG-1-1	Pro-AGG	chr16:3241989-3242060:+	50	2743	303	253
tRNA-Pro-TGG-1-1	Pro-TGG	chr14:21101165-21101236:+	59	3210	334	240
tRNA-Pro-TGG-3-1	Pro-TGG	chr5:180615854-180615925:-	59	3283	343	282
tRNA-Ser-AGA-chr11-8	Ser-AGA	chr11:103274966-103275035:+	46	100	1170	217
tRNA-Tyr-GTA-chr1-127	Tyr-GTA	chr1:566376-566441:-	288	182	426	569
tRNA-Tyr-GTA-chr14-8	Tyr-GTA	chr14:32954018-32954083:+	280	163	398	527
tRNA-Tyr-GTA-chr21-2	Tyr-GTA	chr21:10492972-10493037:-	288	182	426	569
tRNA-Val-CAC-10-1	Val-CAC	chr1:17006501-17006573:-	1	149	71	14

DISCUSSION

Enhanced protein synthesis and proliferation are properties of tumor cells. Even though the translation machinery components were found to be dysregulated in cancer, the role of tRNAs has not been established well. Recently, genome-wide expressions of differential tRNAs in breast cancer were investigated using high throughput technologies. Functional and phenotypic characterization has been performed by transcriptome analyses. Therefore, tRNAs are believed to play important roles in the regulation and progression of breast cancer and they can be excepted as promising prognostic biomarkers (11).

In our study we investigated the MYC-dependent deregulated expressions of tRNAs in breast cancer cells by using next-generation sequencing and bioinformatic analysis revealed that 6 out of 71 tRNAs were nuclear-encoded mt-tRNAs.

In the tRNA nomenclature, tRNA-Amino acid refers to the type of tRNA to be charged with an amino acid (28,29). Our results have shown that nmt-tRNA-Gln anticodon CTG and TTG were found to be deregulated. Sangha et al. demonstrated that $tRNA^{Gln}$ showed a median decrease and was downregulated in several cancers (30). We observed that $tRNA^{Ser}$ was deregulated depending on *MYC*. Serine tRNAs (tRNASer) are found to be overexpressed in breast tumors resulting in poor prognosis and an increased risk of recurrence (31).

Our results also demonstrated that upon *MYC* overexpression the level of $tRNA^{Leu}$ was downregulated and vice versa for MYC inhibition. Leucyl-tRNA synthetase was reported as a tumor suppressor in breast cancer (32). Despite the fact that *MYC* amplification is correlated with malignant phenotypes it masks the effect of the tumor suppressor as for $tRNA^{Leu}$.

Although RNAseq approaches have many advantages like the characterization of transcriptomes, there are also some limitations especially in providing an absolute quantification for the transcripts. With the advance of small RNA sequencing,

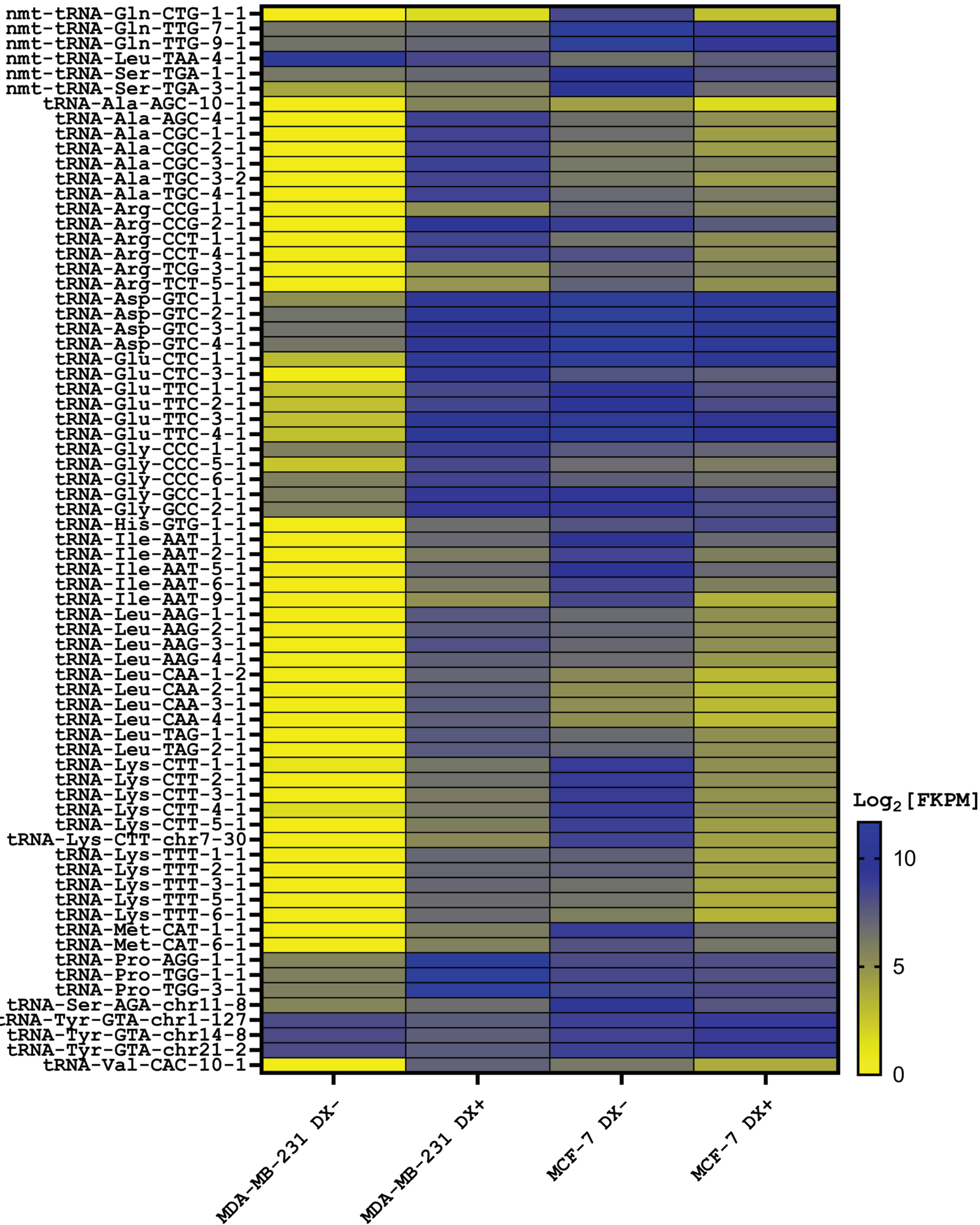

Figure 2: HeatMap Analysis of detected tRNAs in breast cancer cells

it is possible to quantify tRNA gene expression but the limitation is that because of the complexity of tRNA structure sequencing, biases can arise, and also it could be challenging to discriminate reads from mature tRNAs or precursor tRNAs. But nevertheless, small RNA sequencing data are in use for tRNA expressions (33,34).

This study reveals the deregulated expressions of tRNAs including nuclear-encoded mt tRNAs in breast cancer depending on *MYC* overexpression/inhibition. Functional studies are required for underlying molecular mechanisms.

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