# Molecular analysis of long non-coding RNA GAS5 and microRNA-34a expression signature in common solid tumors: A pilot study

Eman A. Toraih[1,2*], Saleh Ali Alghamdi[3], Aya El-Wazir[1,2], Marwa M Hosny[4], Mohammad H. Hussein[5], Motaz S. Khashana[6], Manal S. Fawzy[4,7*]

[1] Genetics Unit, Department of Histology and Cell Biology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

[2] Center of Excellence of Molecular and Cellular Medicine, Suez Canal University, Ismailia, Egypt

[3]Medical Genetics, Clinical Laboratory Department, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia

[4]Department of Medical Biochemistry, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

[5] Pulmonologist, Ministry of Health and Population, Cairo, Egypt

[6] Faculty of Medicine, Suez Canal University, Ismailia, Egypt

[7] Department of Biochemistry, Faculty of Medicine, Northern Border University, Arar, Saudi Arabia.

***Corresponding authors:**

E-mail: manal2_khashana@ymail.com (MSF)

E-mail: emantoraih@gmail.com (EAT)

**Short title:** LncRNA GAS5 and miR-34a expression in cancer

## Abstract

Accumulating evidence indicates that non-coding RNAs including microRNAs (miRs) and long non-coding RNAs (lncRNAs) are aberrantly expressed in cancer, providing promising biomarkers for diagnosis, prognosis and/or therapeutic targets. We aimed in the current work to quantify the expression profile of miR-34a and one of its bioinformatically selected partner lncRNA growth arrest-specific 5 (GAS5) in a sample of Egyptian cancer patients, including three prevalent types of cancer in our region; renal cell carcinoma (RCC), hepatocellular carcinoma (HCC) and glioblastoma (GB) as well as to correlate these expression profiles with the available clinicopathological data in an attempt to clarify their roles in cancer. Quantitative real-time polymerase chain reaction analysis was applied. Different bioinformatics databases were searched to confirm the potential miRNAs-lncRNA interactions of the selected ncRNAs in cancer pathogenesis. GAS5 was significantly under-expressed in the three types of cancer. However, levels of miR-34a greatly varied according to the tumor type; it displayed an increased expression in RCC [4.05 (1.003-22.69), $p$ <0.001] and a decreased expression in GB [0.35 (0.04-0.95), $p$ <0.001]. A weak negative correlation was observed between levels of GAS5 and miR-34a in GB [r = -0.39, $p$ =0.006]. Univariate analyses revealed a correlation of *GAS5* downregulation with poor disease-free survival (r = 0.31, $p$ =0.018) and overall survival (r = 0.28, $p$ =0.029) in RCC but not in GB, and a marginal significance correlation with a higher number of lesions in HCC. Hierarchical clustering analysis showed RCC patients among others, could be clustered by GAS5 and miR-34a co-expression profile. Our results confirm the tumor suppressor role of GAS5 in cancer and suggest its potential applicability to be a predictor of bad outcomes with other conventional markers for various types of cancer. Further functional validation studies are warranted to confirm miR-34a/GAS5 interplay in cancer.

**Keywords:** GAS5; miR-34a; RCC; GB; HCC

## Introduction

Cancer is now the second leading cause of mortality worldwide, causing 8.8 million deaths globally in 2015, which is equivalent to one in every six deaths [1]. Cancer occurs as a net result of activation of oncogenes and inhibition of tumor suppressor genes (TSGs) [2]. Decades back, it was believed that oncogenes and TSGs had to code for proteins, and only mutations in protein-coding genes would result in such pathological conditions as cancer. However, in the world of genetics, it is never that simple. With the advancement of genetic technologies including next generation sequencing, microarrays and bioinformatic machinery, many truths came to light, including what was originally thought to be junk DNA is now found to code for thousands of equally significant regulatory RNAs [3]. Since their discovery, the non-coding RNAs (ncRNAs) have been recognized as epigenetic regulators of protein-coding genes. Recently, a whole new level of regulation has been uncovered, when it was found that ncRNAs have the ability to regulate each other as well [4] further adding to the complexity of the regulatory processes. This set of findings has revolutionized our understanding of several human diseases, making this 'the era of non-coding RNAs'.

Many classes of ncRNAs have been identified and linked to cancer, the most common of which are micro RNAs (miRNAs), long-noncoding RNAs (lncRNAs), PIWI-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs). The roles of these four types of ncRNAs in cancer are reviewed in several studies [5-8]. In brief, overexpression of some ncRNAs as miRNAs or lncRNAs can suppress the expression of TSG targets, while loss of function or reduced expression of others may allow overexpression of the oncogenes they regulate. Furthermore, since each ncRNA may regulate hundreds of different genes, its over or under expression may have widespread oncogenic effects because many genes will be dysregulated [9].

Here we were interested in a relatively newly discovered lncRNA; Growth Arrest Specific 5 (GAS5) which is a poorly conserved gene mapped to chromosome 1q25.1 [10]. It consists of 12 exons and 11 introns from which 29 transcripts are produced from alternative splicing, many of which contain retained introns (Ensembl Genome Browser 'GAS5'). The first two transcripts

discovered (produced from alternative splice sites on exon 7) are the GAS5a and GAS5b with the latter being predominantly expressed in most cells [11]. Another synonym for the gene is' Small Nucleolar RNA Host Gene', this is for having the ability to produce multiple (10 in human) non-coding small nucleolar RNAs (snRNAs) from its more conserved introns [11]. These snRNAs are involved in the regulation of ribosomal RNA (rRNA) synthesis through 2-O-methylation of pre-ribosomal RNA [12].

As its name implies, GAS5 is over expressed in growth-arrested cells [13]. This is further demonstrated by the presence of high levels of GAS5 in brain cells, which are considered the slowest dividing cells in the body as opposed to its lowest levels in other rapidly dividing cells, the most important of which are cancer cells [14].

Expectedly, GAS5 under expression was found to be associated with multiple types of cancer [15-25] and it was found to bind some miRNAs, including miR-21, miR-222 and miR-103 sponging their inhibitory effect on their target TSGs ([16, 21, 26, 27].

Our bioinformatic analyses have revealed a potential new miRNA target for the GAS5 gene; miR-34a. This miRNA is encoded from chromosome1p36.22. Its promotor is recognized for having multiple CpG islands and a p53 binding site, making p53 a direct transcriptional regulator for this miRNA [28, 29]. While the history of miR-34a with cancer is very well established, studies conducted on different types of cancers have contradictory results regarding its actual role in tumor progression. In other words, is it an oncogene or a TSG? Many studies have proved it's functioning as a TSG in various types of cancer, including neuroblastoma [30], leukemia [31], pancreatic [32] and hepatocellular [33, 34] carcinoma, glioblastoma (GB) [35, 36], breast [37] lung [38] and colon [39] cancer. On the contrary, other studies have found that it functions as an oncogene through promoting tumorigenesis as in RCC [40, 41], papillary thyroid carcinoma (PTC) [42], colon [39] and uterine cancer [43]. It has been suggested that this discrepancy may be attributed to the tissue type and the miR-34a/p53 pathway involved [41].

To the best of our knowledge, no clinical studies were conducted to explore both GAS5 and miR-

34a profiles in cancer patients. Hence, we were interested to investigate the expression profiles of these ncRNAs in three prevalent types of cancer in our region; renal cell carcinoma (RCC), hepatocellular carcinoma (HCC) and glioblastoma (GB) as well as to correlate these expression profiles with the available clinicopathological data in an attempt to clarify their roles in cancer.

## Materials and Methods

### Genomic characterization of GAS5 and miR-34a

Chromosomal localization, genomic sequence and structure analysis, subcellular localization, variant analysis, and folding pattern were retrieved from different online tools; including Ensembl (http://www.ensembl.org/), GeneCards for human gene database (http://www.genecards.org/), National Center for Biotechnology Information (NCBI) (ncbi.nlm.nih.gov/), COMPARTMENTS subcellular localization (https://compartments.jensenlab.org/Search), Database of Transcription Start Sites (DBTSS) version 10.0 (http://dbtss.hgc.jp/), KineFold (http://kinefold.curie.fr/), and MFold webserver (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form).

### Exploring miR-34 and GAS5 interactions

Identifying complementary regions between microRNA-34a and lncRNA GAS5 were demonstrated by several tools; RNA22 microRNA target detection (https://cm.jefferson.edu/rna22/) and DIANA-LncBase v2 (http://diana.imis.athena-innovation.gr/DianaTools/index.php).

### Functional enrichment analysis

Pathway enrichment analysis and gene ontology of microRNA-34a was performed by Diana-miRPath v3.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php) using its experimentally validated gene targets. Functional enrichment analysis of GAS5 was obtained from Ensembl and GeneCards databases to identify its biological function in cancer.

### Study population and sampling collection

In total, 230 samples were analyzed, including 60 formalin-fixed, paraffin-embedded (FFPE) RCC samples and their paired adjacent non-cancer tissues, 50 FFPE GB specimens and 10 non-cancer brain tissues as well as 30 HCC blood samples and 20 controls.

131 ***(a) RCC cohort***

132 The archived FFPE renal samples were taken from sixty patients who underwent radical
133 nephrectomy for a primary RCC and dating back for three years. All retrieved cases were archived
134 in the Pathology laboratory of Mansoura Oncology Center, Mansoura, Egypt. No history of
135 receiving neoadjuvant chemotherapy or radiotherapy prior to sampling. Clinicopathological data,
136 including the survival were collected from patient medical records. Paired sixty cancer-free adjacent
137 tissues were examined and sectioned to serve as controls for molecular analysis.

138 ***(b) GB cohort***

139 Fifty glioblastoma patients and 10 non-cancer brain tissues collected from the archive of the
140 Pathology Department, Mansoura University Hospitals, Egypt, dating back for three years were
141 included in the current work. Detailed patients' data were retrieved from their follow up records.
142 They had GB grade 4, undergone surgical removal and had not received any treatment before
143 sampling. They followed-up for more than 3 years.

144 ***(c) HCC cohort***

145 Following our local hospital and medical ethical committee rules in liver tissue sampling
146 prohibition from HCC patients, only blood samples were available. Thirty HCV-induced HCC and
147 20 matched controls from healthy blood bank donors were recruited in the study. Patients were
148 obtained from the outpatient clinic of Tropical Medicine and Gastroenterology Department, Faculty
149 of Medicine, Assuit, Egypt. They had typical imaging findings of liver cancer and elevated alpha
150 fetoprotein (AFP). Patients underwent clinical and radiological assessment, confirmation of HCV
151 by PCR, Barcelona-Clinic Liver Cancer (BCLC) staging, and Child-Turcotte-Pugh (CTP) scoring
152 (44). Survival data for HCC patients were not available in patients' medical records. Hence, these
153 data were not included in the statistical analysis for HCC patients.

154 **Ethical approval**

155 The study was conducted according to the ethical guidelines of the Declaration of Helsinki and
156 approved by the Medical Research Ethics Committee of Suez Canal University.

**RNA extraction**

Total RNA; including the small RNA, was isolated from either FFPE tissue sections (5 to 8-µm-thick) using the Qiagen miRNeasy FFPE Kit (*Qiagen, cat no 217504*) or serum using Qiagen miRNeasy serum/plasma Kit (*Qiagen, cat no 217184*) following the protocols supplied by the manufacturer. Concentration of RNA was determined using the NanoDrop ND-1000 spectrophotometer (*NanoDrop Tech., Inc. Wilmington, DE, USA*). Samples with a 260/280 nm absorbance ratio less than 1.8 were excluded.

**Reverse transcription reaction**

Subsequently, RNA for lncRNA GAS5 was converted to complementary DNA (cDNA) in a T-Professional Basic, Biometra PCR System (*Biometra, Goettingen, Germany*) using high Capacity cDNA Reverse Transcription Kit (*Applied Biosystems, P/N 4368814*) with RT random primers as previously described [45].

Reverse transcription of miR-34a was specifically converted to cDNA using TaqMan™ MicroRNA Reverse Transcription kit (*P/N 4366596; Applied Biosystems, Foster City, CA, USA*) with 5x miRNA specific stem–loop primers as previously described in our prior publication [46]. Appropriate controls were included in each experiment.

**LncRNA GAS5 and microRNA-34a expression analyses**

The Real-Time PCR reactions were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [47]. The expression level of *GAS5* was assessed via SYBR Green qPCR analysis and normalized with GAPDH. The following primers were designed using Primer 3 software and checked by *in silico* PCR amplification of the University of California, Santa Cruz (UCSC) genome browser; for *GAS5*: Forward: 5′- CTTGCCTGGACCAGCTTAAT-3′; Reverse: 5′-CAAGCCGACTCTCCATACCT-3′, and for *GAPDH*: Forward: 5'- CGGATTTGGTCGTATTGGG-3'; Reverse: 5'-CTGGAAGATGGTGATGGGATT-3'. In brief, 10 µl of qPCR Green Master (*Jena Bioscience, Cat no. PCR-313L*), 0.6 µl (10 µM) forward and reverse primers, 8 µl PCR grade water, and 1 µl

cDNA template were included in the reaction for *GAS5* and *GAPDH* SYBR Green assay analyses [46]. For microRNA quantification, a final volume of 20 µl was adjusted in duplicate, including 1.33 µl specific RT products, 2× TaqMan® Universal PCR Master Mix with UNG (Applied Biosystems, P/N 4440043), and 20× of 1 µl specific TaqMan® RNA assay for hsa-miR-34a-5p (Applied Biosystems, assay ID 000426) and Taqman® Universal PCR master mix II, No UNG (2×) [48]. Two endogenous control assays were used; TATA box binding protein (TBP; assay ID Hs00427620_m1) in brain cancer and RNU6B (assay ID Hs001093) in liver and renal cancer [49, 50]. Appropriate negative and positive controls were used. The PCR for 96-well plates was carried out using StepOne™ Real-Time PCR System (Applied Biosystem) and incubated as follows: 95°C for 10 min followed by 45 cycles of 92°C for 15 seconds and 60°C for 1 minute. Ten percent randomly selected study samples were re-evaluated in separate runs for the study gene expressions to test the reproducibility of the qPCR which showed very close quantitative cycles value results and low standard deviations.

**Statistical analysis**

R package (version 3.3.2) and Statistical Package for the Social Sciences (SPSS) for Windows software (version 22.0) were used for data analyses. Categorical variables were compared using the Chi-square ($\chi^2$) or Fisher's exact tests where appropriate. Wilcoxon matched-pair signed-rank and Mann-Whitney U tests were used for tissues of renal/brain cancer and serum of liver cancer patients, respectively, to compare continuous variables. The correlation between miR-34a level and *GAS5* expressions was calculated by Spearman's rank correlation analysis. A two-tailed *p*-value of $< 0.05$ was considered statistically significant. The receiver operating characteristic (ROC) curves were performed to get the best cutoff values of miRNA-34a for discriminating long and short survivors in cancer patients. The fold change of ncRNAs expressions in each patient relative to the control was calculated via Livak method based on the quantitative cycle (Cq) values with the following equation: relative quantity = $2^{-\Delta\Delta Cq}$; where $\Delta\Delta C_q = (C_q \text{ ncRNA} - C_q \text{ internal control})_{\text{cancer}} - (C_q \text{ ncRNA} - C_q \text{ internal control})_{\text{control}}$ [51]. Univariate analysis for association between ncRNA

expression profile and clinico-pathological features in cancer patients was run. The software package named PC-ORD ver. 6 [52] was employed to run different multivariate analyses for clustering analysis of patients according to clinico-pathological and molecular data.

## Results

### Genomic location of GAS5

Lnc-RNA GAS5 is also known as small nucleolar RNA host gene 2 (SNHG2) and non-protein coding RNA 30 (NCRNA00030). It is encoded by GAS5 gene on chromosome 1q25.1 (Genomic coordination at 1:173863901-173867987 at the negative strand according to human genome assembly GRCh38) (**Fig. 1A**). It consists of 12 exons, spanning 4.087 kb and encoding for 29 different alternative splice transcripts ranging in length from 242 to 1698 bp (Electronic Supplementary Table S1).

### Sequence analysis of *GAS5*

*GAS5* gene contains a seven-nucleotide oligo-pyrimidine tract on its 5′-end in exon 1, hence is classified as a member of the 5' terminal oligo-pyrimidine (TOP) genes. This sequence can act as a cis-regulatory motif which either inhibits the binding of translational regulatory proteins downstream to the transcriptional start sites of mRNAs or suppresses the translational machinery itself. In addition to its translational controls, the TOP elements are known to modulate gene expression through regulating transcription [53]. Being one of the TOP genes, it is ubiquitously expressed and is predicted to regulate the translation of more than 20% of total mRNAs [DataBase of Transcription Start Sites]

Sequence analysis of *GAS5* gene revealed that it is a small nucleolar RNA host gene, containing multiple *snoRNA* genes within its introns. These genes encode for ten C/D box snoRNAs, which contain the C (UGAUGA) and D (CUGA) box motifs. They are predicted to play a role in the 2'-O-methylation of rRNA by guiding guanine methylation, which enhances RNA folding and interaction with ribosomal proteins. *GAS5* also hosts SNORDA103 within intron 4, an H/ACA box snoRNA, which is associated with pseudouridylation (**Fig. 1B**).

## Variant analysis

*GAS5* gene is shown to be highly polymorphic, enclosing around 300 thousand variants (75% intronic, 21% exonic, and 4% splice region polymorphisms). Among all these polymorphisms, 14 SNPs, one deletion and one insertion are common variants with minor allele frequency (MAF) > 0.05 (**Fig. 1C**) and (Electronic Supplementary Table S2).

**Fig. 1 Structural analysis of *GAS5* gene.** (A) Chromosomal localization of *GAS5* gene. It is present on chromosome 1q25.1, at genomic coordination 1:173863901-173867987 on the negative strand (according to human genome assembly GRCh38). (B) Sequence analysis of *GAS5* gene. It consists of 12 exons, spanning 4.087 kb that code for 29 different alternative splice transcripts. It hosts multiple snoRNA genes within its introns (except intron 9). These genes encode for ten C/D box snoRNAs, which contain the C (UGAUGA) and D (CUGA) box motifs, and an H/ACA box snoRNA, SNORDA103, within intron 4. (C) Genetic variant analysis. *GAS5* gene (4087 bases long) contains around 300 thousand variants (75% intronic, 21% exonic, and 4% splice region polymorphisms). Among all these polymorphisms, 14 SNPs (red), one deletion (green) and one insertion (blue) are common variants with minor allele frequency (MAF) > 0.05. (D) Subcellular localization of lncRNA GAS5. Text mining highlighted its predominant existence intranuclear and within extracellular exosomes which are extruded into the circulation. (F) Folding pattern of lncRNA GAS5. [Data source: Ensembl.org, genecards.org, NCBI, COMPARTMENT database, and MFold]

## GAS5-miRNA interaction

Analysis with the RNA22 program (http://cbcsrv.watson.ibm.com/rna22.html) identified complementary regions of GAS5 with 690 microRNAs. Among these putative microRNAs, only 252 interactions with 234 microRNAs showed significant binding ($p<0.05$) (Electronic Supplementary Table S3). Via DIANA-LncBase v2 database for experimentally validated miRNA-

lncRNA interactions, GAS5 was identified as a miR-34a target by immunoprecipitation experiments (score=0.558). For further confirmation, we used RNA22 software to determine the interaction binding sites between GAS5 transcripts and miR-34a. Our results showed base-pairing in twenty-three alternative splice variants. Among them, nine transcripts had two miR-34a binding sites (Electronic Supplementary Table S4).

**miR-34a functional analysis**

Hundreds of miR-34a-5p and 3p targets were retrieved from various online databases; including miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), miRDB (http://mirdb.org/) and microRNA.org. Functional enrichment analysis revealed its enrollment in cancer-related KEGG pathways, as Pathways in cancer (hsa05200, 115 targets, p=0.001723304), proteoglycans in cancer (hsa05205, 71 targets, p=1.687731e-06), adherens junction (hsa04520, 34 targets, $p$ = 3.396929e-06), cell cycle (hsa04110, 54 targets, $p$ = 3.355808e-05), and p53 signaling pathway (hsa04115, 31 targets, $p$ = 0.006060223) as well as cancer-specific pathways, namely glioma (hsa05214, 29 targets, $p$ = 0.0003111577) and renal cell carcinoma (hsa05211, 28 targets, p= 0.02521455) (Electronic Supplementary Table S5). GO analysis (Diana tools) demonstrated miR-34a to be involved in cell death, cell cycle, and response to stress thus highlighting its role in cancer cell growth. In addition, miR-34a was significantly associated with membrane organization, cell junction organization, and cell motility, hence may play a key role in cancer cell invasion and metastasis (**Fig. 2**).

**Fig. 2 Functional and structural analysis of miR-34a.** (A) GAS5: miR-34a-5p interaction. Complementarity regions are shown in three binding sites in *GAS5* gene, one proximal at 5' region (1432-1455) and two distal at 3' end (3545-3567 and 3698-3719) [Data source: RNA22, DIANA-LncBase v2]. (B) Predicted secondary structure of miR-34a. Folding pattern and energy are demonstrated [Data source: KineFold]. (C) Functional enrichment analysis of miR-34a experimentally validated gene targets. Significant clustered heat map represents the gene ontology using GO slim option, FDR conservative states, $p$ value threshold < 0.05, and categories union

[Data source: DIANA-miRPath v3]. http://snf-515788.vm.okeanos.grnet.gr/uploads/R/HeatMap290913.png

## Characteristics of the study population

Baseline characteristics of RCC, GB, and HCC cohorts are demonstrated in **Tables 1** to **3**.

**Table 1. Clinicopathological characteristics of renal cell carcinoma patients**

| Variables | Total | Low OS | High OS | *p* value |
|---|---|---|---|---|
| **Total number** | 60 (100) | 19 (31.7) | 41 (68.3) | |
| **Age** | | | | |
| ≤55 years | 6 (10.0) | 3 (15.8) | 3 (7.3) | 0.370 |
| >55 years | 54 (90.0) | 16 (84.2) | 38 (92.7) | |
| **Gender** | | | | |
| Female | 21 (35.0) | 9 (47.4) | 1 (29.3) | 0.245 |
| Male | 39 (65.0) | 10 (52.6) | 29 (70.7) | |
| **HPD** | | | | |
| Clear cell | 30 (50.0) | 8 (42.1) | 22 (53.7) | 0.354 |
| Papillary | 15 (25.0) | 4 (21.1) | 11 (26.8) | |
| Chromophobic | 15 (25.0) | 7 (36.8) | 8 (19.5) | |
| **Tumor location** | | | | |
| Right side | 22 (36.7) | 7 (36.8) | 15 (36.6) | 0.985 |
| Left side | 38 (63.3) | 12 (63.2) | 26 (63.4) | |
| **Grade** | | | | |
| Grade 1 | 9 (15.0) | 1 (5.3) | 8 (19.5) | **0.023** |
| Grade 2 | 28 (46.7) | 6 (31.6) | 22 (53.7) | |
| Grade 3 | 23 (38.3) | 12 (63.2) | 11 (26.8) | |
| **Tumor size** | | | | |
| T1 | 21 (35.0) | 6 (31.6) | 15 (36.6) | 0.589 |
| T2 | 25 (41.7) | 7 (16.8) | 18 (43.9) | |
| T3 | 14 (23.3) | 6 (31.6) | 8 (19.5) | |
| **LN** | | | | |
| Negative | 39 (65.0) | 11 (57.9) | 28 (68.3) | 0.562 |
| Positive | 21 (35.0) | 8 (42.1) | 13 (31.7) | |
| **Recurrence** | | | | |
| Negative | 44 (73.3) | 7 (36.8) | 37 (90.2) | **<0.001** |
| Positive | 16 (26.7) | 12 (63.2) | 4 (9.8) | |
| **DFS** | | | | |
| ≤ 1year | 29 (48.3) | 19 (100) | 10 (24.4) | **<0.001** |
| > 1year | 31 (51.7) | 0 (0.0) | 31 (75.6) | |
| **GAS5 fold** | | | | |
| ≤ 1-fold | 55 (91.7) | 19 (100) | 36 (87.8) | 0.283 |
| > 1-fold | 3 (5.0) | 0 (0.0) | 3 (7.3) | |
| >10-folds | 2 (3.3) | 0 (0.0) | 2 (4.9) | |

| | | | | |
|---|---|---|---|---|
| **miR-34a fold** | | | | |
| ≤ 1-fold | 15 (25.0) | 6 (31.6) | 9 (22.0) | 0.192 |
| > 1-fold | 19 (31.7) | 8 (42.1) | 11 (26.8) | |
| >10-folds | 26 (43.3) | 5 (26.3) | 21 (51.2) | |

*HPD* histopathological diagnosis, *DFS* disease-free survival, *OS* overall survival. Low OS: ≤12 months, High OS: >12 months. Fisher's Exact and Pearson Chi-square tests were used. Bold values indicate statistical significance at $p < 0.05$

**Table 2. Clinicopathological characteristics of glioblastoma patients**

| Variables | Total | Low OS | High OS | *p* value |
|---|---|---|---|---|
| **Total number** | 50 (100) | 10 (20.0) | 40 (80.0) | |
| **Age** | | | | |
| ≤55 years | 21 (42.0) | 7 (70.0) | 14 (35.0) | 0.073 |
| >55 years | 29 (58.0) | 3 (3.0) | 26 (65.0) | |
| **Gender** | | | | |
| Female | 13 (26.0) | 1 (10.0) | 12 (30.0) | 0.258 |
| Male | 37 (74.0) | 9 (90.0) | 28 (70.0) | |
| **Tumor location** | | | | |
| Frontal | 24 (48.0) | 8 (80.0) | 16 (40.0) | 0.071 |
| Tempero-paroetal | 18 (36.0) | 1 (10.0) | 17 (42.5) | |
| Fronto-temporal | 8 (16.0) | 1 (10.0) | 7 (17.5) | |
| **Recurrence** | | | | |
| Negative | 44 (88.0) | 8 (80.0) | 36 (90.0) | 0.586 |
| Positive | 6 (12.0) | 2 (20.0) | 4 (10.0) | |
| **DFS** | | | | |
| ≤ 1year | 25 (50.0) | 10 (100) | 15 (37.5) | **0.001** |
| > 1year | 25 (50.0) | 0 (0.0) | 25 (62.5) | |
| **GAS5 fold** | | | | |
| ≤ 1-fold | 37 (74.0) | 7 (70.0) | 30 (75.0) | 0.707 |
| > 1-fold | 13 (26.0) | 3 (30.0) | 10 (25.0) | |
| >10-folds | | | | |
| **miR-34a fold** | | | | |
| ≤ 1-fold | 38 (76.0) | 8 980.0) | 30 (75.0) | 0.741 |
| > 1-fold | 12 (24.0) | 2 (20.0) | 10 (25.0) | |
| >10-folds | 0 (0.0) | | | |

Data are presented as number (frequency). *HPD* histopathological diagnosis, *DFS* disease-free survival, *OS* overall survival. Low OS: ≤12 months, High OS: >12 months. Fisher's Exact and Pearson Chi-square tests were used. Bold values indicate statistical significance at $p < 0.05$

**Table 3. Clinicopathological characteristics of hepatocellular carcinoma patients**

| Variables | Patients (n=30) |
|---|---|
| **Total number** | |
| **Age** | |
| ≤55 years | 13 (43.3) |
| >55 years | 17 (56.7) |
| **Gender** | |
| Female | 11 (367) |
| Male | 19 (63.3) |
| **Tumor size** | |
| <5 cm | 9 (30.0) |
| ≥5 cm | 21 (70.0) |
| **Number of lesions** | |
| Solitary | 20 (66.7) |
| Multiple | 10 (33.3) |
| **LCF score** | 9.7±1.2 |
| **CTP class** | |
| Child B | 21 (70.0) |
| Child C | 9 (30.0) |
| **Hepatomegaly** | |
| Mild | 18 (60.0) |
| Massive | 12 (40.0) |
| **Treatment** | |
| Ablation | 15 (30.0) |
| Radiofrequency | 11 (36.7) |
| Supportive | 4 (13.3) |
| **GAS5 fold** | |
| ≤ 1-fold | 27 (90.0) |
| > 1-fold | 3 (10.0) |
| **miR-34a fold** | |
| ≤ 1-fold | 19 (63.3) |
| > 1-fold | 10 (33.3) |

Data are presented as number (frequency). *CTP* Child-Turcotte-Pugh classification for liver cell failure, *LCF* liver cell failure score by CTP classification. Fisher's Exact and Pearson Chi-square tests were used.

## Expression profiling

GAS5 levels were under-expressed in RCC [0.08 (0.006-0.38), $p$ <0.001], GB [0.10 (0.003-0.89), $p$ < 0.001] and HCC [0.12 (0.015-0.74), $p$ < 0.001]. On the other hand, miR-34a displayed an increased expression in RCC [4.05 (1.003-22.69), $p$ < 0.001] and a decreased expression in GB [0.35 (0.04-0.95), $p$ < 0.001] as depicted in **Fig. 3**. A weak negative correlation was observed between levels of GAS5 and miR-34a in GB [r = -0.39, $p$ = 0.006] (**Fig. 4**).

**Fig. 3 Relative expression of GAS5 and miR-34a-5p in cancer.** *RCC* renal cell carcinoma, *GB* glioblastoma, *HCC* hepatocellular carcinoma. Data are represented as medians. The box defines upper and lower quartiles (25% and 75%, respectively) and the error bars indicate upper and lower adjacent limits. Expression levels in cancer and control samples were normalized to GAPDH in RCC and HCC, TBP in GB and RNU6B for microRNA and calculated using the delta-delta CT method [= 2 (-ΔΔCT)] in comparison to controls. Fold change of controls were set at 1.0. Wilcoxon matched-pair signed-rank and Mann-Whitney U tests were used for tissues of renal/brain cancer and serum of liver cancer patients, respectively. Two-sided $p < 0.05$ was considered statistically significant

**Fig. 4 Correlation between GAS5 and miR-34a-5p expression levels.** *RCC* renal cell carcinoma, *GB* glioblastoma, *HCC* hepatocellular carcinoma. Spearman's rank correlation test was used. Statistical significance was considered at $p < 0.05$.

**Association of GAS5 and miR-34a with clinicopathological features**

Univariate analyses are shown in **Table 4A**, B, and C. The lower GAS5 level was correlated with poor DFS (r = 0.31, $p = 0.018$) and OS (r = 0.28, $p = 0.029$) in RCC but not in GB, and was correlated by a marginal significance (r= -0.35, $p = 0.056$) with a higher number of lesions in HCC. Levels of miR-34a did not correlate with any clinico-pathological features.

**Table 4. Univariate analysis for association between gene profile and clinico-pathological features in the study cohorts**

| | (a) RCC | | | |
|---|---|---|---|---|
| | GAS5 | | miR-34a | |
| | $p$ ass | r ($p$ corr) | $p$ ass | r ($p$ corr) |
| **Age** | 0.239 | -0.069 (0.598) | 0.352 | -0.066 (0.619) |
| **Gender** | 0.631 | 0.063 (0.635) | 0.798 | -0.033(0.801) |
| **Location** | 0.908 | -0.015 (0.910) | 0.884 | 0.019 (0.886) |
| **HPD** | 0.257 | 0.191 (0.143) | 0.504 | -0.130(0.321) |
| **Grade** | 0.402 | 0.171 (0.192) | 0.378 | -0.167 (0.204) |
| **Tumor size** | 0.199 | 0.157 (0.230) | 0.361 | -0.152 (0.246) |
| **LN** | 0.193 | 0.169 (0.195) | 0.258 | -0.147(0.261) |
| **Recurrence** | 0.192 | 0.170 (0.195) | 0.371 | -0.116 (0.376) |
| **DFS** | 0.120 | **0.305 (0.018)** | 0.363 | 0.215 (0.099) |
| **OS** | 0.266 | **0.283 (0.029)** | 0.441 | 0.191 (0.144) |
| | **(b) GBM** | | | |
| **Age** | 0.883 | 0.063 (0.663) | 0.930 | -0.013 (0.931) |
| **Gender** | 0.921 | -0.014 (0.922) | 0.514 | -0.093 (0.520) |
| **Location** | 0.530 | -0.150 (0.300) | 0.711 | 0.091 (0.531) |
| **Recurrence** | 0.258 | 0.166 (0.248) | 0.070 | -0.260 (0.068) |
| **DFS** | 0.869 | 0.012 (0.936) | 0.677 | 0.074 (0.609) |
| **OS** | 0.451 | 0.48 (0.743) | 0.356 | 0.013 (0.927) |
| | **(c) HCC** | | | |
| **Age** | 0.869 | -0.037 (0.848) | 0.059 | -0.289 (0.121) |
| **Gender** | 0.553 | 0.112 (0.556) | 0.420 | 0.156 (0.411) |
| **Hepatomegaly** | 0.692 | -0.079 (0.680) | 0.851 | 0.039 (0.837) |
| **Tumor size** | 0.226 | 0.278 (0.137) | 0.209 | -0.315 (0.090) |
| **Number of lesions** | 0.193 | -0.352 (0.056) | 0.916 | 0.112 (0.225) |
| **CTP class** | 0.283 | -0.206 (0.275) | 0.533 | 0.122 (0.521) |
| **Treatment** | 0.715 | 0.144 (0.447) | 0.598 | 0.006 (0.973) |

*p* ass *p* values for association, *r* (*p* corr) correlation spearman's coefficient and *p* value of correlation, *HPD* histopathological diagnosis, *T* tumor size, *LN* lymph node, *DFS* disease-free survival, *OS* overall survival, *CTP* Child-Turcotte-Pugh classification for liver cell failure. Spearman's rank, Mann-Whitney U and Kruskal-Wallis tests were used. Statistically significant values ($p < 0.05$) are shown in bold.

**Role of GAS5 and miR-34a as markers of cancer prognosis**

ROC curves showed no prognostic value for GAS5 nor miR-34a to predict survival of cancer patients in RCC and GB, or to predict CTP class for liver cell failure in HCC patients ($p > 0.05$) (**Fig. 5**).

**Fig. 5 Receiver Operating Characteristics (ROC) analysis for the prognostic value of markers.** *RCC* renal cell carcinoma, *GB* glioblastoma, *HCC* hepatocellular carcinoma, *AUC* area under curve, *CTP* Child-Turcotte-Pugh classification for liver cell failure

**Survival analysis in RCC and GB**

In RCC, multivariable analysis using logistic regression test (Enter method) showed age and pathological grade to be independent predictors for recurrence: (OR = 1.251, 95% CI = 1.075-1.455, $p$ = 0.004) for the age and (OR = 19.9, 95% CI = 1.034-383, $p$ = 0.047) for the grade. Kaplan-Meier curve analysis and log-rank test revealed that RCC patients with female gender, post-nephrectomy recurrence, advanced pathological grade, and down-regulated miR-34a levels had significantly poor overall survival than their corresponding ($p < 0.05$) (**Table 5**). In addition, multivariable analysis by Cox regression model demonstrated gender and recurrence to be independent predictors of overall survival (hazard ratio (HR) = 2.49; 95 % confidence interval (95 % CI) 1.14–5.41, $p = 0.021$) and (HR = 4.16; 95% CI of 1.88–9.16, $p < 0.001$), respectively.

In GB, Kaplan-Meier curves showed a significant association of shorter survival times with male gender (Breslow test: $p$ = 0.002 and Tarane-Ware test: $p$ = 0.030). In addition, marginal significance was observed for poor overall survival in patients with frontal lesions (Log rank test: $p$ = 0.050) (**Table 6**). Multivariable analysis by Cox regression model illustrated tumor recurrence to be an independent predictor of low overall survival (HZ = 11.1; 95 % CI 2.88-42.5, $p < 0.001$).

**Table 5. Multivariable analysis in renal cell carcinoma patients**

| Variables | Survival time | Overall comparisons | | | Cox regression | |
|---|---|---|---|---|---|---|
| | OS (mo) | Log Rank | Breslow | Tarone-Ware | HR (95% CI) | *p* |
| **Age** | | | | | | |
| ≤55 years | 13.1 ± 2.22 | | | | | |
| >55 years | 16.1 ± 0.8 | 0.186 | 0.244 | 0.221 | 0.97 (0.92-1.01) | 0.217 |
| **Gender** | | | | | | |
| Female | 13.9 ± 1.28 | | | | | |
| Male | 16.7 ± 0.94 | **0.027** | 0.077 | 0.095 | **2.49 (1.14-5.41)** | **0.021** |
| **HPD** | | | | | | |
| Type 1 | 16.1 ± 1.12 | | | | | |
| Type 2 | 17.5 ± 1.61 | | | | 0.70 (0.34-1.46) | 0.351 |
| Type 3 | 13.3 ± 1.25 | 0.103 | 0.198 | 0.159 | 0.69 (0.30-1.60) | 0.397 |
| **Location** | | | | | | |
| Right side | 16.3 ± 1.23 | | | | | |
| Left side | 15.4 ± 1.00 | 0.703 | 0.489 | 0.539 | 1.08 (0.57-2.06) | 0.807 |
| **Grade** | | | | | | |
| Grade 1 | 16.3 ± 1.53 | | | | | |
| Grade 2 | 18.0 ± 1.17 | | | | | |
| Grade 3 | 12.8 ± 1.05 | **0.005** | **0.003** | **0.003** | 1.60 (0.66-3.85) | 0.293 |
| **Tumor size** | | | | | | |
| T1 | 16.4 ± 1.40 | | | | | |
| T2 | 15.9 ± 1.13 | | | | 0.91 (0.30-2.75) | 0.871 |
| T3 | 14.5 ± 1.65 | 0.709 | 0.577 | 0.635 | 0.89 (0.34-2.29) | 0.813 |
| **LN** | | | | | | |
| Negative | 16.4 ±0.97 | | | | | |
| Positive | 14.6 ± 1.27 | 0.306 | 0.235 | 0.255 | 1.05 (0.42-2.59) | 0.911 |
| **Recurrence** | | | | | | |
| Negative | 17.5 ± 0.83 | | | | | |
| Positive | 10.8 ± 1.07 | **<0.001** | **<0.001** | **<0.001** | **4.16 (1.88-9.16)** | **<0.001** |
| **GAS5 fold** | | | | | | |
| Under-expressed | 15.7 ± 0.84 | | | | | |
| Over-expressed | 16.2 ± 1.24 | 0.735 | 0.753 | 0.985 | 0.29 (1.04-0.96) | 0.291 |
| **miR-34a fold** | | | | | | |
| Under-expressed | 13.2 ± 0.99 | | | | | |
| Over-expressed | 16.6 ± 0.95 | **0.010** | **0.049** | **0.024** | 1.001 (0.98-1.01) | 0.926 |

Survival times is shown as mean and standard error, *OS* overall survival, *HR (95% CI)* Hazard ratio (95% confidence interval), *HPD* histopathological diagnosis, *T* tumor size, *LN* lymph node,. Statistically significant values ($p < 0.05$) are shown in bold.

391 **Table 6. Multivariable analysis in glioblastoma patients**
392

| Variables | Survival time | Overall comparisons | | | Cox regression | |
|---|---|---|---|---|---|---|
| | OS (mo) | Log Rank | Breslow | Tarone-Ware | HR (95% CI) | *p* |
| **Age** | | | | | | |
| ≤55 years | 17.8 ± 1.4 | 0.742 | 0.683 | 0.595 | 1.03 (0.97-1.09) | 0.298 |
| >55 years | 17.0 ± 0.96 | | | | | |
| **Gender** | | | | | | |
| Female | 20.5 ± 1.5 | 0.096 | **0.002** | **0.030** | 1.36 (0.45-4.11) | 0.583 |
| Male | 16.2 ± 0.8 | | | | | |
| **Tumor location** | | | | | | |
| Frontal | 15.7 ± 1.01 | **0.050** | 0.079 | 0.062 | | 0.749 |
| Tempero-paroetal | 17.6 ± 1.3 | | | | 1.3 (053-3.39) | 0.534 |
| Fronto-temporal | 21.5 ± 2.1 | | | | 1.5 (0.44-5.75) | 0.472 |
| **Recurrence** | | | | | | |
| Negative | 17.6 ± 0.8 | 0.284 | 0.397 | 0.342 | **11.1 (2.88-42.5)** | **<0.001** |
| Positive | 15.3 ± 1.8 | | | | | |
| **GAS5 fold** | | | | | | |
| Under-expressed | 17.5 ± 0.9 | 0.765 | 0.581 | 0.642 | 096 (0.49-1.85) | 0.904 |
| Over-expressed | 16.9 ± 1.6 | | | | | |
| **miR-34a fold** | | | | | | |
| Under-expressed | 17.7 ± 0.9 | 0.460 | 0.453 | 0.415 | 1.14 (0.79-1.65) | 0.471 |
| Over-expressed | 16.1 ± 1.5 | | | | | |

393
394 Survival time is shown as mean and standard error, *OS* overall survival, *HR (95% CI)* Hazard ratio
395 (95% confidence interval). Statistically significant values ($p < 0.05$) are shown in bold.

396

397 **Hierarchical clustering analysis**

398 Dendrograms for Two-way agglomerative hierarchical cluster analysis, were employed (**Fig. 6**).
399 The following cluster setup parameters were adjusted: Distance method: Sorensen (Bray-Curtis),
400 Group Linkage Method: Flexible Beta at 0.75, Clustering of factor relative by factor maximum. A
401 distance matrix is shown. RCC plot analyzed 60 strands and 12 factors, GB plot analyzed 50 strands
402 and 9 factors, whereas HCC dendrogram showed results of 30 strands and 11 factors. Clustering
403 analysis revealed separation of RCC patients by GAS5 and miR-34a levels, GB patients by survival
404 times, and HCC patients by their age.

405

406

407

**Fig. 6 Multivariate analysis of patients according to combined transcriptomic signature of genes and clinicopathological features. (A) RCC, (B) GB, (C) HCC.** RCC patients were divided based on GAS5 and miR-34a levels, GB patients were divided by survival times, and HCC patients by age

## Discussion

In this study, we measured the expression of two ncRNAs; the lncRNA GAS5 and the miRNA miR-34a in three of the most prevalent and high-incidence tumors in Egypt; hepatic, renal and brain cancer. We chose more than one tumor type to assess if the same ncRNA could work differently according to the tissue type. We also investigated the possible association between GAS5 and miR-34a in mediating carcinogenesis after detecting an interaction between the two through our preliminary in *silico* analysis.

Our results show that *GAS5* was under-expressed in the three types of cancer; RCC, HCC and GB. On the other hand, levels of miR-34a greatly varied according to the tumor type. RCC patients had a lower GAS5 level and a higher miR-34a level in tumor tissue compared to adjacent normal tissue. Moreover, GAS5 levels were correlated with poor survival. In accordance, Qiao et al. reported a reduced GAS5 level in RCC cell lines compared to normal cell lines. Furthermore, in *vitro* cloning and functional expression analysis revealed that *GAS5* overexpression caused cell cycle arrest, increased apoptosis, as well as inhibited tumor metastatic potential [24], which could explain the correlation between reduced patient survival and GAS5 level in our study. As for miR-34a, several studies reported over-expression of this RNA in RCC patients [40, 41, 54-56]. Liu et al. predicted that its oncogenic function could be through targeting the two tumor suppressors secreted frizzled related protein 1 (SFRP1) and calmodulin binding transcription activator 1 (CAMTA1), and further validated the first target [40]. Another study noticed that miR-34a enhances cell survival, both in *vitro* and in *vivo* during cisplatin nephrotoxicity through p53 [57]. Collectively, the previous studies suggest that miR-34a can act as an oncogene in renal tissue. On the contrary, Yadav et al. detected

434 a significant under-expression of miR-34a in both sera and renal tissues of RCC patients [58].
435 Similar results were demonstrated by the studies of Zhang et al. and Weng et al. [59, 60], where the
436 former study demonstrated that decreased expression of miR-34a in RCC patients inversely up-
437 regulated the gene for the transcription factor YY1. The latter study suggested that under-
438 expression of miR-34a in cancer tissues of RCC patients affected the regulation of the *NOTCH1*
439 gene, as well as caused dysregulation of other multiple miR-34a targets in 786-O and Caki-1 RCC
440 cell lines. Yu et al. found that miR-34a suppressed tumor growth and metastasis in *vivo* and in *vitro*
441 through targeting CD44 [61]. Another study found that miR-34a inhibits cellular invasion in renal
442 cancer cell lines A498 and 769P through targeting the 3' untranslated region (UTR) of the *c-myc*
443 oncogene [62]. Also, the lncRNA NEAT1 (nuclear paraspeckle assembly transcript 1) was found to
444 sponge miR-34a releasing its inhibition on the *c-met* oncogene, resulting in increased cellular
445 proliferation and invasion in RCC cell lines 786-O and ACHN [63]. Furthermore, Bai et al. found
446 that miR-34a causes senescence of rat renal cells through targeting two anti-oxidative mitochondrial
447 genes [64]. Cellular senescence, which is an irreversible state of growth arrest, protects the cells
448 from accumulating mutations that could lead to malignant transformation [65]. Further supporting
449 the tumor suppressor potential of miR-34a, Zhou et al. provided evidence that miR-34a, secreted by
450 fibroblasts, enhances apoptosis of renal tubular cells through regulating the anti-apoptotic gene
451 *BCL-2* [66].
452 The inconsistent results between our study which shows up-regulation of *miR-34a* in RCC and
453 other studies showing its under-regulation in the same type of cancer could have many possible
454 explanations. First, we measured the expression of miR-34a in cancer tissues obtained directly from
455 RCC patients as opposed to other studies conducted on cancer cell lines. While those cell lines are
456 highly essential for functional molecular analysis, they may be different from primary tumors,
457 possibly through building up new mutations in their attempt to adjust to their artificial environment
458 [67, 68]. Such mutations may easily alter cellular responses and regulatory mechanisms, possibly
459 affecting the expression of *miR-34a*. Second, miR-34a could be a non-specific molecule that can

460 both activate or inhibit tumerogenesis depending on the surrounding environment. These include
461 internal stimuli (other regulatory molecules or polymorphisms, oxidative molecules, other
462 associated disease states, tumor stage/grade or else) and external stimuli (cellular response to
463 environmental exposures, including chemotherapy, other drugs, chemicals, foods, etc). Third, miR-
464 34a has multiple targets (discussed in our previous work) [41], as well as being itself a target for
465 many lncRNAs. Each study focuses on one or few targets, and infers a tumor suppressor or an
466 oncogenic function based on its effect on the studied target/pathway. However, miR-34a is one
467 small molecule in a larger network of molecules that either promotes or inhibits tumorogenesis
468 based on the net result of *all* its regulated targets, which could easily differ according to countless
469 variables. Given by the negative correlation of this microRNA with GAS5 in our RCC patients, as
470 well as the predicted interaction between the two, we believe that a new pathway; the *GAS5*/miR-
471 34a pathway might be involved in the previously indicated molecular network leading to RCC.
472 The interaction between lncRNAs and miRNAs can be multifaceted. Yoon et al. explained four
473 mechanisms of interaction between the two types [69]. First, lncRNAs can act as miRNA sponges
474 as previously mentioned with examples in the introduction. Second, the opposite can occur, where
475 miRNAs can inhibit lncRNAs by binding to them and causing their degradation. This applies for
476 miR145-5p, miR-181a-5p and miR-99b-3p which inhibit lncRNA ROR [70], and miR-9 which
477 inhibits lncRNA MALAT1 [71]. Third, miRNAs and lncRNAs can both compete for the same
478 binding site on mRNAs, an example of which is miR-485-5p and lncRNA BACE1AS competing
479 for BACE1 mRNA [72]. Fourth and finally, another form of relationship exists between the two
480 types; where the lncRNA (>200 nucleotide) is capable of generating smaller (<22 nucleotide)
481 miRNAs such as the lncRNA H19 generating miR-675 [73]. GAS5 provides a perfect example for
482 such interactions. For instance, GAS5 acts as a molecular sponge for many different microRNAs,
483 including miR-21, miR-222, miR-196a, miR-205, miR-221 and miR-103. [16, 26, 74-77], all of
484 which are related to cancer. Then again, both miR-21 and miR-222 can negatively regulate *GAS5*
485 [27, 78]. Also, three of the snoRNAs produced by GAS5 (U44, U74 and U78) can give rise to

miRNAs [79], making GAS5 one of the lncRNAs generating miRNAs.

While functional validation has been yet required to prove the direct interaction between GAS5 and miR-34a, it is highly plausible. This is due to the already verified interaction between miR-34a and GAS1 (Growth arrest specific 1), another member of the *GAS* genes [42]. *GAS1* is a protein coding gene that, similar to *GAS5*, exerts its tumor suppressor actions through arresting the cell cycle and stimulating apoptosis [80]. Ma et al. measured the expression of miR-34a and the GAS1 protein in papillary thyroid carcinoma; GAS1 was under-expressed, while miR-34a was over-expressed. Further analysis revealed that miR-34a binds to the 3'UTR of GAS1 causing its silencing, which in turn activates the *RET* oncogene [42]. Through the BLAST tool, we detected sequence homology between the *GAS1* and the *GAS5* genes, further raising the possibility of interaction of miRNA-34a with GAS5. Given that *GAS5* is down-regulated and *miR-34a* is up-regulated in our study, tumorogenesis may be in such a case due to sponging of miR-34a by GAS5, where under-expression of *GAS5* in the rapidly dividing cancer cells causes release of the inhibition of miR-34a on its tumor suppressor targets, allowing for further tumor progression. Oppositely, the inverse correlation between the two could also suggest that miR-34a inhibits GAS5, making its under-expression a cause of cancer rather than a result, as in the case of the miR-34a -GAS1 interaction.

miR-34a expression levels in our study were not significant in the case of patients with HCC. *GAS5* levels, however, were under-expressed in HCC patients. In addition, lower GAS5 levels were associated with more numerous tumor foci. The same conclusions were reached by studies conducted by Tu et al., Chang et al. and Hu et al. who found that *GAS5* was under-expressed in HCC patients and predicted poor survival in those patients [15, 26, 81]. The latter study by Hu et al. found that under-expression of *GAS5* in HCC cell lines releases its sponging effect on oncomiR-21, which normally targets the two tumor suppressor genes *PDCD4* and *PTEN* [26]. On the contrary, according to Tao et al., *GAS5* was a proto-oncogene in HCC where they found an indel polymorphism in the promotor of *GAS5* that increased the risk of HCC in Chinese. In *vitro* analysis showed that the deletion allele of the polymorphism altered methylation of the *GAS5* promoter and

was associated with higher levels of GAS5 in HCC cell lines Sk-hep-1, Bel-7404 and Huh7. Further analysis provided evidence that the resulting over-expression of *GAS5* had an anti-apoptotic effect in those cell lines [82]. This divergence observed by Tao et al. could be due to racial differences. In other words, the allele causing *GAS5* over-expression might be more common in their studied population. To further confirm this probability, we searched the 1000 genome project phase 3 databases [83] for this indel polymorphism (positon 1:173868254-173868258 (AGGCA/-)). We found that the frequency of the deletion allele was very high in Asians and Chinese in particular (28-40%) as compared to other races (3-12%), showing that in other populations the effect of the polymorphism on HCC and *GAS5* expression could be negligible.

In patients with GB, both GAS5 and miR-34a levels were significantly down-regulated. Zhang et al. correlated the expression of five lncRNAs including GAS5 with GB, and found that higher levels of GAS5 were associated with prolonged survival [84]. Zhao et al. found that *GAS5* was under-expressed in glioma cell lines U87 and U251 and that its tumor suppressor role was through targeting miR-222 [16]. The same author, in a more recent study, added another miRNA to the targets of GAS5; miR-196a-5p, where *GAS5* under-expression in human glioma stem cells enhanced tumor progression through inhibiting this miRNA [85]. Regarding the more controversial *miR-34a*, studies on brain cancer, including GB and glioma agree on its role as a tumor suppressor in this particular tissue; reviewed in [86], which fits in with its expression status in the present study.

## Conclusions

In this study, we show that *GAS5* is under-expressed in three types of tumors, in addition to being associated with tumor prognosis in some types. Consequently, we believe that GAS5 could potentially be used as a prognostic marker for cancer. Furthermore, in favor of this notion is the predicted subcellular localization of GAS5 provided by our in *silico* analysis, which shows that GAS5 is most significantly localized in the extracellular exosomes, supporting its existence in the circulation and highlighting its putative role as a non-invasive biomarker which mirrors tissue

pathology. Furthermore, we suppose that miR-34a might be a potential target of GAS5, or *vice versa*. However, we confirm that further studies are required to validate this interaction.

**Acknowledgements**

The authors thank the Center of Excellence in Molecular and Cellular Medicine and the Oncology Diagnostic Unit, Suez Canal University, Ismailia, Egypt for providing the facilities for performing the research work.

**Compliance with ethical standards**

**Conflicts of interest**

The authors declare that they have no competing interests.

**Funding**

None.

**References**

[1] WHO | Cancer [WWW Document], World Health Organization. Available from URL http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed 9.2.18).

[2] Grandér D. How do mutated oncogenes and tumor suppressor genes cause cancer? Med Oncol. 1998; 15:20–6. https://doi.org/10.1007/BF02787340

[3] ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489:57–74. https://doi.org/10.1038/nature11247

[4] Paraskevopoulou MD, Hatzigeorgiou AG. Analyzing MiRNA–LncRNA Interactions, in: Long Non-Coding RNAs, Methods in Molecular Biology. Humana Press, New York, NY, 2016; pp. 271–286. https://doi.org/10.1007/978-1-4939-3378-5_21

[5] Bartonicek N, Maag JLV, Dinger ME. Long noncoding RNAs in cancer: mechanisms of action and technological advancements. Mol Cancer. 2016; 15:43. https://doi.org/10.1186/s12943-016-0530-6

[6] Leva GD, Garofalo M, Croce CM. MicroRNAs in Cancer. Annu Rev Pathol Mech Dis. 2014; 9:287–314. https://doi.org/10.1146/annurev-pathol-012513-104715

564 [7] Mannoor K, Liao J, Jiang F. Small nucleolar RNAs in cancer. Biochim Biophys Acta. 2012;
565 1826. https://doi.org/10.1016/j.bbcan.2012.03.005
566 [8] Ng KW, Anderson C, Marshall EA, Minatel BC, Enfield KSS, Saprunoff HL, et al. Piwi-
567 interacting RNAs in cancer: emerging functions and clinical utility. Mol Cancer. 2016; 15:5.
568 https://doi.org/10.1186/s12943-016-0491-9
569 [9] Lo P-K, Wolfson B, Zhou X, Duru N, Gernapudi R, Zhou Q. Noncoding RNAs in breast
570 cancer. Brief Funct Genomics. 2016; 15: 200–21. https://doi.org/10.1093/bfgp/elv055
571 [10] Raho G, Barone V, Rossi D, Philipson L, Sorrentino V. The gas 5 gene shows four
572 alternative splicing patterns without coding for a protein. Gene. 2000; 256:13–7.
573 https://doi.org/10.1016/S0378-1119(00)00363-2
574 [11] Smith CM, Steitz JA. Classification of gas5 as a Multi-Small-Nucleolar-RNA (snoRNA)
575 Host Gene and a Member of the 5′-Terminal Oligopyrimidine Gene Family Reveals
576 Common Features of snoRNA Host Genes. Mol Cell Biol. 1998;18:6897-909.
577 [12] Maden BE, Hughes JM. Eukaryotic ribosomal RNA: the recent excitement in the
578 nucleotide modification problem. Chromosoma. 1997; 105:391–400.
579 [13] Schneider C, King RM, Philipson L. Genes specifically expressed at growth arrest of
580 mammalian cells. Cell. 1988; 54:787–93.
581 [14] Coccia EM, Cicala C, Charlesworth A, Ciccarelli C, Rossi G B, Philipson L, et al.
582 Regulation and expression of a growth arrest-specific gene (gas5) during growth,
583 differentiation, and development. Molecular and Cellular Biology. 1992; 12:3514-21.
584 [15] Chang L, Li C, Lan T, Long Wu, Yufeng Yuan, Quanyan Liuet, al. Decreased expression
585 of long non-coding RNA GAS5 indicates a poor prognosis and promotes cell proliferation
586 and invasion in hepatocellular carcinoma by regulating vimentin. Mol Med Rep. 2016;
587 13:1541-50. https://doi.org/10.3892/mmr.2015.4716
588 [16] Xihe Zhao, Ping Wang, Jing Liu, Jian Zheng, Yunhui Liu, Jiajia Chen et al. Gas5 Exerts
589 Tumor-suppressive Functions in Human Glioma Cells by Targeting miR-222. Mol Ther.

23:1899–911. https://doi.org/10.1038/mt.2015.170

[17] Wenjun Liang, Tangfeng Lv, Xuefei Shi, Hongbing Liu, Qingqing Zhu, Junli Zeng, et al. Circulating long noncoding RNA GAS5 is a novel biomarker for the diagnosis of nonsmall cell lung cancer. Medicine (Baltimore). 2016; 95:e4608. https://doi.org/10.1097/MD.0000000000004608

[18] Cao Q, Wang N, Qi J, Gu Z, Shen H. Long non-coding RNA-GAS5 acts as a tumor suppressor in bladder transitional cell carcinoma via regulation of chemokine (C-C motif) ligand 1 expression. Mol Med Rep. 2016; 13:27–34. https://doi.org/10.3892/mmr.2015.4503

[19] Yacqub-Usman K, Pickard MR, Williams GT. Reciprocal regulation of GAS5 lncRNA levels and mTOR inhibitor action in prostate cancer cells. The Prostate 2015; 75:693–705. https://doi.org/10.1002/pros.22952

[20] Pickard MR, Williams GT 2014. Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy. Breast Cancer Res Treat. 2014; 145:359–70. https://doi.org/10.1007/s10549-014-2974-y

[21] Guo C, Song W-Q, Sun P, Jin L, Dai H-Y. LncRNA-GAS5 induces PTEN expression through inhibiting miR-103 in endometrial cancer cells. J. Biomed. Sci. 2015; 22:100. https://doi.org/10.1186/s12929-015-0213-4

[22] Lu X, Fang Y, Wang Z, Xie J, Zhan Q, Deng X, et al. Downregulation of gas5 increases pancreatic cancer cell proliferation by regulating CDK6. Cell Tissue Res. 2013; 354:891–6. https://doi.org/10.1007/s00441-013-1711-x

[23] Yin D, He X, Zhang E, Kong R, De W, Zhang Z. Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. Med Oncol Northwood Lond Engl. 2014; 31:253. https://doi.org/10.1007/s12032-014-0253-8

[24] Qiao H-P, Gao W-S, Huo J-X, Yang Z-S. Long Non-coding RNA GAS5 Functions as a Tumor Suppressor in Renal Cell Carcinoma. Asian Pac J Cancer Prev. 2013; 14: 1077–82. https://doi.org/10.7314/APJCP.2013.14.2.1077

616 [25] Nakamura Y, Takahashi N, Kakegawa E, Yoshida K, Ito Y, Kayano H, et al. The GAS5
617 (growth arrest-specific transcript 5) gene fuses to BCL6 as a result of t(1;3) (q25;q27) in a
618 patient with B-cell lymphoma. Cancer Genet. Cytogenet. 2008; 182:144–9.
619 https://doi.org/10.1016/j.cancergencyto.2008.01.013
620 [26] Hu L, Ye H, Huang G, Luo F, Liu Y, Liu Y, et al. Long noncoding RNA GAS5
621 suppresses the migration and invasion of hepatocellular carcinoma cells via miR-21.
622 Tumour Biol. 2016; 37:2691–702. https://doi.org/10.1007/s13277-015-4111-x
623 [27] Zhang Z, Zhu Z, Watabe K, Zhang X, Bai C, Xu M, et al. Negative regulation of lncRNA
624 GAS5 by miR-21. Cell Death Differ. 2013; 20:1558–68.
625 https://doi.org/10.1038/cdd.2013.110
626 [28] He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of
627 the p53 tumour suppressor network. Nature. 2007; 447:1130–4.
628 https://doi.org/10.1038/nature05939
629 [29] Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Körner H, et al.
630 Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell
631 Cycle. 2008; 7:2591–600. https://doi.org/10.4161/cc.7.16.6533
632 [30] Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor
633 by inducing apoptosis in neuroblastoma cells. Oncogene. 2007; 26:5017–22.
634 https://doi.org/10.1038/sj.onc.1210293
635 [31] Chim CS, Wong KY, Qi Y, Loong F, Lam WL, Wong LG, et al. Epigenetic inactivation
636 of the miR-34a in hematological malignancies. Carcinogenesis. 2010; 31: 745–50.
637 https://doi.org/10.1093/carcin/bgq033
638 [32] Ji Q, Hao X, Zhang M, Tang W, Yang M, Li L, et al. MicroRNA miR-34 inhibits human
639 pancreatic cancer tumor-initiating cells. PloS One. 2009; 4:e6816.
640 https://doi.org/10.1371/journal.pone.0006816
641 [33] Li N, Fu H, Tie Y, Hu Z, Kong W, Wu Y, et al. miR-34a inhibits migration and invasion

642 by down-regulation of c-Met expression in human hepatocellular carcinoma cells. Cancer
643 Lett. 2009; 275:44–53. https://doi.org/10.1016/j.canlet.2008.09.035
644 [34] Shehata RH, Abdelmoneim SS, Osman OA, Hasanain AF, Osama A, Abdelmoneim SS, et
645 al. Deregulation of miR-34a and Its Chaperon Hsp70 in Hepatitis C virus-Induced Liver
646 Cirrhosis and Hepatocellular Carcinoma Patients. Asian Pacific Journal of Cancer
647 Prevention. APJCP. 2017;18(9):2395-2401. doi:10.22034/APJCP.2017.18.9.2395
648 [35] Li Y, Guessous F, Zhang Y, Dipierro C, Kefas B, Johnson E, et al. MicroRNA-34a
649 inhibits glioblastoma growth by targeting multiple oncogenes. Cancer Res. 2009; 69: 7569–
650 76. https://doi.org/10.1158/0008-5472.CAN-09-0529
651 [36] Toraih EA, Aly NM, Abdallah HY, Al-Qahtani SA, Shaalan AA, Hussein MH, et al.
652 MicroRNA-target cross-talks: Key players in glioblastoma multiforme. Tumour Biol.
653 2017;39(11):1010428317726842. https://doi.org/10.1177/1010428317726842
654 [37] Kato M1, Paranjape T, Müller RU, Nallur S, Gillespie E, Keane K, et al. The mir-34
655 microRNA is required for the DNA damage response in vivo in C. elegans and in vitro in
656 human breast cancer cells. Oncogene. 2009; 28:2419–24.
657 https://doi.org/10.1038/onc.2009.106
658 [38] Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D, et al. Development
659 of a lung cancer therapeutic based on the tumor suppressor microRNA-34. Cancer Res.
660 2010; 70:5923–30. https://doi.org/10.1158/0008-5472.CAN-10-0655
661 [39] Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. Tumor-suppressive miR-34a induces
662 senescence-like growth arrest through modulation of the E2F pathway in human colon
663 cancer cells. Proc Natl Acad Sci USA. 2007; 104:15472–7.
664 https://doi.org/10.1073/pnas.0707351104
665 [40] Liu H, Brannon AR, Reddy AR, Alexe G, Seiler MW, Arreola A, et al. Identifying mRNA
666 targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell
667 Carcinoma. BMC Syst Biol. 2010; 4:51. https://doi.org/10.1186/1752-0509-4-51

668 [41] Toraih EA, Ibrahiem AT, Fawzy MS, Hussein MH, Al-Qahtani SAM, Shaalan AAM.
669 MicroRNA-34a: A Key Regulator in the Hallmarks of Renal Cell Carcinoma. Oxid Med
670 Cell Longev. 2017; 2017:3269379. https://doi.org/10.1155/2017/3269379
671 [42] Ma Y, Qin H, Cui Y. MiR-34a targets GAS1 to promote cell proliferation and inhibit
672 apoptosis in papillary thyroid carcinoma via PI3K/Akt/Bad pathway. Biochem Biophys Res
673 Commun. 2013; 441:958–63. https://doi.org/10.1016/j.bbrc.2013.11.01043. Tazawa et al.,
674 2007
675 [43] Marsh EE1, Lin Z, Yin P, Milad M, Chakravarti D, Bulun SE. Differential expression of
676 microRNA species in human uterine leiomyoma versus normal myometrium. Fertil Steril.
677 2008; 89:1771–6. https://doi.org/10.1016/j.fertnstert.2007.05.074
678 [44] Llovet JM, Fuster J, Bruix J. Barcelona-Clínic Liver Cancer Group. The Barcelona
679 approach: diagnosis, staging, and treatment of hepatocellular carcinoma. Liver Transpl.
680 2004; 10:S115-20. doi: 10.1002/lt.20034
681 [45] Toraih EA, Fawzy MS, El-Falouji AI, Elham O Hamed, Nader A Nemr, Mohammad H
682 Hussein, et al. Stemness-Related Transcriptional Factors and Homing Gene Expression
683 Profiles in Hepatic Differentiation and Cancer. Mol Med. 2016; 22:653-63.
684 https://doi.org/10.2119/molmed.2016.00096
685 [46] Toraih EA, Fawzy MS, Mohammed EA, Hussein MH, El-Labban MM. MicroRNA-196a2
686 Biomarker and Targetome Network Analysis in Solid Tumors. Mol Diagn Ther. 2016;
687 20:559-77. https://doi.org/10.1007/s40291-016-0223-2
688 [47] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE
689 guidelines: minimum information for publication of quantitative real-time PCR experiments.
690 Clin Chem. 2009; 55:611–22. https://doi.org/10.1373/clinchem.2008.112797
691 [48] Toraih EA, Mohammed EA, Farrag S, Ramsis N, Hosny S. Pilot Study of Serum
692 MicroRNA-21 as a Diagnostic and Prognostic Biomarker in Egyptian Breast Cancer
693 Patients. Mol Diagn Ther. 2015; 19:179-90. https://doi.org/10.1007/s40291-015-0143-6.

694 [49] Valente V, Teixeira SA, Neder L, Okamoto OK, Oba-Shinjo SM, Marie SK, et al.
695 Selection of suitable housekeeping genes for expression analysis in glioblastoma using
696 quantitative RT-PCR. Ann Neurosci. 2014; 21(2):62-3.
697 https://doi.org/10.5214/ans.0972.7531.210207
698 [50] Aithal MGS, Rajeswari N. Validation of Housekeeping Genes for Gene Expression
699 Analysis in Glioblastoma Using Quantitative Real-Time Polymerase Chain Reaction. Brain
700 Tumor Res Treat. 2015; 3:24-29. https://doi.org/10.14791/btrt.2015.3.1.24.
701 [51] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
702 quantitative PCR and the 2 (-Delta Delta C (T)) Method. Methods. 2001, 25:402–8.
703 https://doi.org/10.1006/meth.2001.1262
704 [52] McCune B, Mefford MJ. PC-ORD. Multivariate Analysis of Ecological Data. Version 6.
705 MjM Software, Gleneden Beach, Oregon, USA, 2011.
706 [53] Yamashita R, Suzuki Y, Takeuchi N, Wakaguri H, Ueda T, Sugano S, et al.
707 Comprehensive detection of human terminal oligo-pyrimidine (TOP) genes and analysis of
708 their characteristics. Nucleic Acids Res. 2008; 36:3707-15.
709 https://doi.org/10.1093/nar/gkn248
710 [54] Cheng T, Wang L, Li Y, Huang C, Zeng L, Yang J. Differential microRNA expression in
711 renal cell carcinoma. Oncol Lett. 2013; 6:769–76. https://doi.org/10.3892/ol.2013.1460
712 [55] Munari E, Marchionni L, Chitre A, Hayashi M, Martignoni G, Brunelli M, et al. Clear cell
713 papillary renal cell carcinoma: micro-RNA expression profiling and comparison with clear
714 cell renal cell carcinoma and papillary renal cell carcinoma. Hum Pathol. 2014; 45:1130–8.
715 https://doi.org/10.1016/j.humpath.2014.01.013
716 [56] Fritz HK, Gustafsson A, Ljungberg B, Ceder Y, Axelson H, Dahlbäck B. The Axl-
717 Regulating Tumor Suppressor miR-34a Is Increased in ccRCC but Does Not Correlate with
718 Axl mRNA or Axl Protein Levels. PloS One. 2015; 10:e0135991.
719 https://doi.org/10.1371/journal.pone.0135991

720 [57] Bhatt K, Zhou L, Mi QS, Huang S, She JX, Dong Z. MicroRNA-34a is induced via p53
721 during cisplatin nephrotoxicity and contributes to cell survival. Mol Med. 2010; 16:409–16.
722 https://doi.org/10.2119/molmed.2010.00002
723 [58] Yadav S, Khandelwal M, Seth A, Saini AK, Dogra PN, Sharma A. Serum microRNA
724 Expression Profiling: Potential Diagnostic Implications of a Panel of Serum microRNAs for
725 Clear Cell Renal Cell Cancer. Urology. 2017; 104:64–9.
726 https://doi.org/10.1016/j.urology.2017.03.013
727 [59] Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, et al. Long noncoding RNA
728 ANRIL indicates a poor prognosis of gastric cancer and promotes tumor growth by
729 epigenetically silencing of miR-99a/miR-449a. Oncotarget. 5:2276–92.
730 https://doi.org/10.18632/oncotarget.1902
731 [60] Weng W, Wang M, Xie S, Long Y, Li F, Sun F, et al. YY1-C/EBPα-miR34a regulatory
732 circuitry is involved in renal cell carcinoma progression. Oncol Rep. 2014; 31:1921–7.
733 https://doi.org/10.3892/or.2014.3005
734 [61] Yu G, Li H, Wang J, Gumireddy K, Li A, Yao W, et al. miRNA-34a Suppresses Cell
735 Proliferation and Metastasis by Targeting CD44 in Human Renal Carcinoma Cells. J Urol.
736 2014; 192:1229–37. https://doi.org/10.1016/j.juro.2014.05.094
737 [62] Yamamura S, Saini S, Majid S, Hirata H, Ueno K, Chang I, et al. MicroRNA-34a
738 suppresses malignant transformation by targeting c-Myc transcriptional complexes in
739 human renal cell carcinoma. Carcinogenesis. 2012; 33:294–300.
740 https://doi.org/10.1093/carcin/bgr286
741 [63] Liu F, Chen N, Gong Y, Xiao R, Wang W, Pan Z. The long non-coding RNA NEAT1
742 enhances epithelial-to-mesenchymal transition and chemoresistance via the miR-34a/c-Met
743 axis in renal cell carcinoma. Oncotarget. 2017; 8:62927–38.
744 https://doi.org/10.18632/oncotarget.17757
745 [64] Bai XY, Ma Y, Ding R, Fu B, Shi S, Chen XM. miR-335 and miR-34a Promote Renal

746 Senescence by Suppressing Mitochondrial Antioxidative Enzymes. J Am Soc Nephrol.
747 2011; 22:1252–61. https://doi.org/10.1681/ASN.2010040367
748 [65] Collado M, Blasco MA, Serrano M. Cellular Senescence in Cancer and Aging. Cell. 2007;
749 130:223–33. https://doi.org/10.1016/j.cell.2007.07.003
750 [66] Zhou Y, Xiong M, Niu J, Sun Q, Su W, Zen K, et al. Secreted fibroblast-derived miR-34a
751 induces tubular cell apoptosis in fibrotic kidney. J Cell Sci. 2014; 127:4494–506.
752 https://doi.org/10.1242/jcs.155523
753 [67] Borrell B. How accurate are cancer cell lines? Nature. 2010; 463:858.
754 https://doi.org/10.1038/463858a
755 [68] Clark MJ, Homer N, O'Connor BD, Chen Z, Eskin A, Lee H, et al. U87MG Decoded: The
756 Genomic Sequence of a Cytogenetically Aberrant Human Cancer Cell Line. PLoS Genet.
757 2010; 6:e1000832. https://doi.org/10.1371/journal.pgen.1000832
758 [69] Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, et al. LincRNA-
759 p21 suppresses target mRNA translation. Mol Cell. 2012; 47:648–55.
760 https://doi.org/10.1016/j.molcel.2012.06.027
761 [70] Wang Y, Xu Z, Jiang J, Xu C, Kang J, Xiao L, et al. Endogenous miRNA Sponge
762 lincRNA-RoR Regulates Oct4, Nanog, and Sox2 in Human Embryonic Stem Cell Self-
763 Renewal. Dev Cell. 2013; 25:69–80. https://doi.org/10.1016/j.devcel.2013.03.002
764 [71] Leucci E, Patella F, Waage J, Holmstrøm K, Lindow M, Porse B, et al. microRNA-9
765 targets the long non-coding RNA MALAT1 for degradation in the nucleus. Sci Rep. 2013;
766 3:2535. https://doi.org/10.1038/srep02535
767 [72] Faghihi MA, Zhang M, Huang J, Modarresi F, Van der Brug MP, Nalls MA, et al.
768 Evidence for natural antisense transcript-mediated inhibition of microRNA function.
769 Genome Biol. 2010; 11: R56. https://doi.org/10.1186/gb-2010-11-5-r56
770 [73] Keniry A1, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G, et al. The H19 lincRNA is
771 a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol.

772 2012; 14:659–65. https://doi.org/10.1038/ncb2521

773 [74] Guo C, Song WQ, Sun P, Jin L, Dai HY. LncRNA-GAS5 induces PTEN expression
774 through inhibiting miR-103 in endometrial cancer cells. J Biomed Sci. 2015; 22:100.
775 https://doi.org/10.1186/s12929-015-0213-4

776 [75] Xue D, Zhou C, Lu H, Xu R, Xu X, He X. LncRNA GAS5 inhibits proliferation and
777 progression of prostate cancer by targeting miR-103 through AKT/mTOR signaling
778 pathway. Tumor Biol. 2016; 37:16187–97. https://doi.org/10.1007/s13277-016-5429-8

779 [76] Yang W, Hong L, Xu X, Wang Q, Huang J, Jiang L. LncRNA GAS5 suppresses the
780 tumorigenesis of cervical cancer by downregulating miR-196a and miR-205. Tumour Biol.
781 2017; 39:1010428317711315. https://doi.org/10.1177/1010428317711315

782 [77] Ye K, Wang S, Zhang H, Han H, Ma B, Nan W. Long Noncoding RNA GAS5 Suppresses
783 Cell Growth and Epithelial-Mesenchymal Transition in Osteosarcoma by Regulating the
784 miR-221/ARHI Pathway. J Cell Biochem. 2017; 118:4772-81.
785 https://doi.org/10.1002/jcb.26145

786 [78] Yu F, Zheng J, Mao Y, Dong P, Lu Z, Li G, et al. Long Non-coding RNA Growth Arrest-
787 specific Transcript 5 (GAS5) Inhibits Liver Fibrogenesis through a Mechanism of
788 Competing Endogenous RNA. J Biol Chem. 2015; 290:28286–98.
789 https://doi.org/10.1074/jbc.M115.683813

790 [79] Brameier M, Herwig A, Reinhardt R, Walter L, Gruber J. Human box C/D snoRNAs with
791 miRNA like functions: expanding the range of regulatory RNAs. Nucleic Acids Res. 2011;
792 39:675–86. https://doi.org/10.1093/nar/gkq776

793 [80] Domínguez-Monzón G, Benítez JA, Vergara P, Lorenzana R, Segovia J. Gas1 inhibits cell
794 proliferation and induces apoptosis of human primary gliomas in the absence of Shh. Int J
795 Dev Neurosci. 2009; 27:305–13. https://doi.org/10.1016/j.ijdevneu.2009.03.009

796 [81] Tu ZQ, Li RJ, Mei JZ, Li XH. Down-regulation of long non-coding RNA GAS5 is
797 associated with the prognosis of hepatocellular carcinoma. Int. J Clin Exp Pathol. 2014;

798 7:4303–9.

799 [82] Tao R, Hu S, Wang S, Zhou X, Zhang Q, Wang C, et al. Association between indel
800 polymorphism in the promoter region of lncRNA GAS5 and the risk of hepatocellular
801 carcinoma. Carcinogenesis. 2015; 36:1136–43. https://doi.org/10.1093/carcin/bgv099

802 [83] The 1000 Genomes Project Consortium. A global reference for human genetic variation.
803 Nature. 2015; 526:68–74. https://doi.org/10.1038/nature15393

804 [84] Zhang XQ, Sun S, Lam KF, Kiang KM, Pu JK, Ho AS, et al. A long non-coding RNA
805 signature in glioblastoma multiforme predicts survival. Neurobiol Dis. 2013; 58:123–31.
806 https://doi.org/10.1016/j.nbd.2013.05.011

807 [85] Zhao X, Liu Y, Zheng J, Liu X, Chen J, Liu L, et al. GAS5 suppresses malignancy of
808 human glioma stem cells via a miR-196a-5p/FOXO1 feedback loop. Biochim Biophys Acta.
809 2017; 1864:1605–17. https://doi.org/10.1016/j.bbamcr.2017.06.020

810 [86] Møller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M. A
811 Systematic Review of MicroRNA in Glioblastoma Multiforme: Micro-modulators in the
812 Mesenchymal Mode of Migration and Invasion. Mol Neurobiol. 2013; 47:131–44.
813 https://doi.org/10.1007/s12035-012-8349-7

814

815 **Supporting Information**

816 **S1 Table: GAS5 alternative splicing transcripts.**

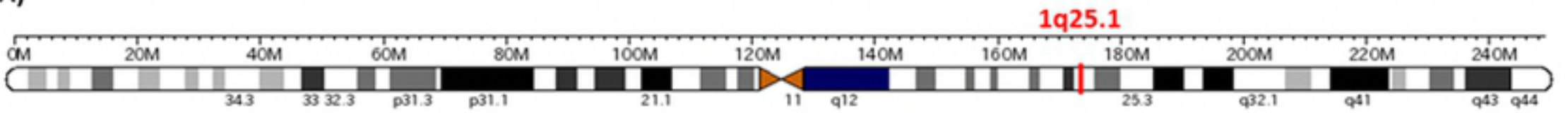
817 **S2 Table: GAS5 common variants.**

818 **S3 Table: LncRNA GAS5-microRNA interaction.**

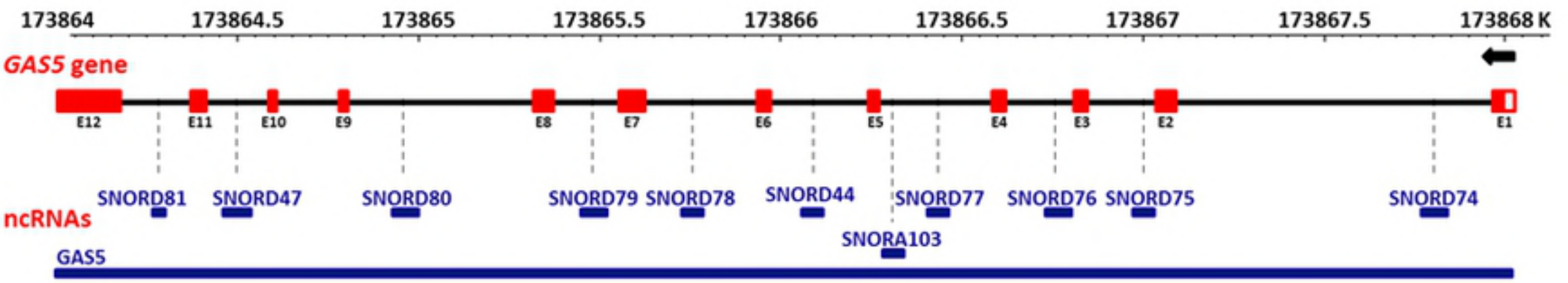
819 **S4 Table: Complementarity between GAS5 transcripts and miR-34a-5p.**

820 **S5 Table: hsa-miR-34a pathways.**

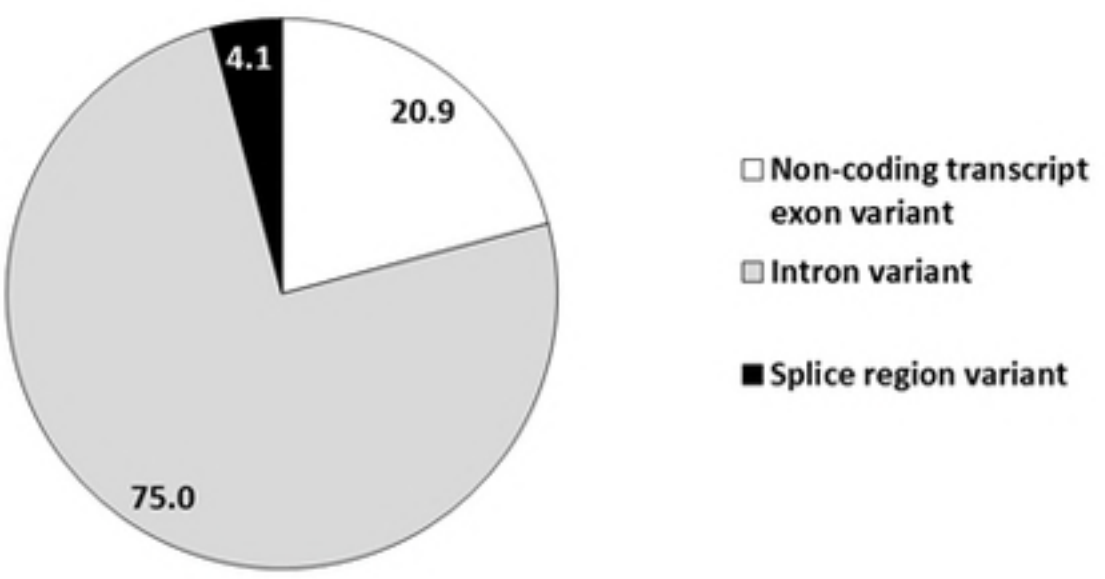
(A)



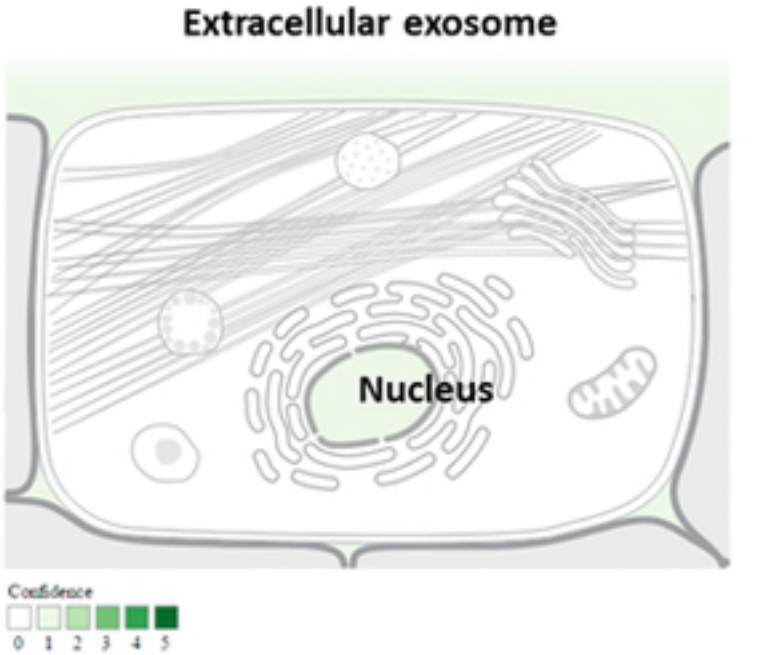

(B)



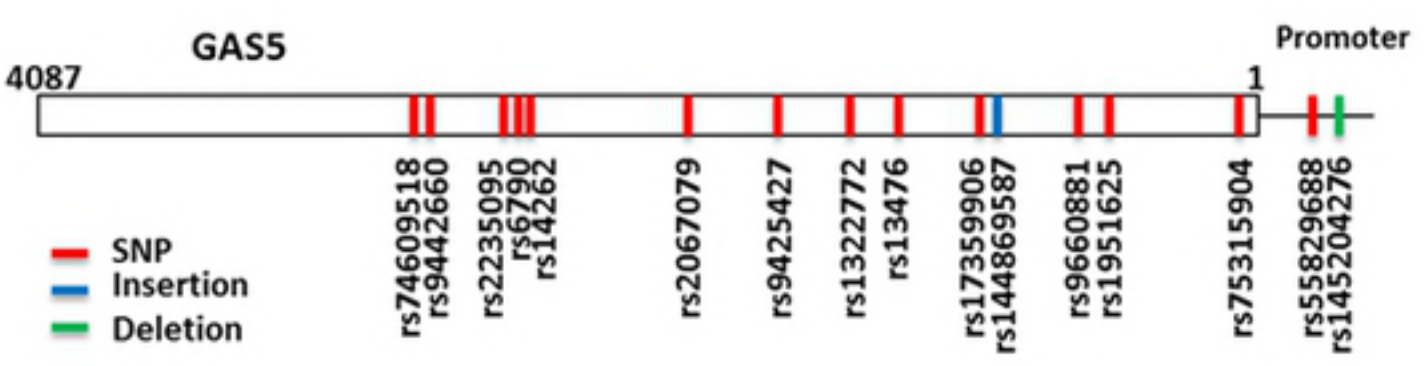

(C)



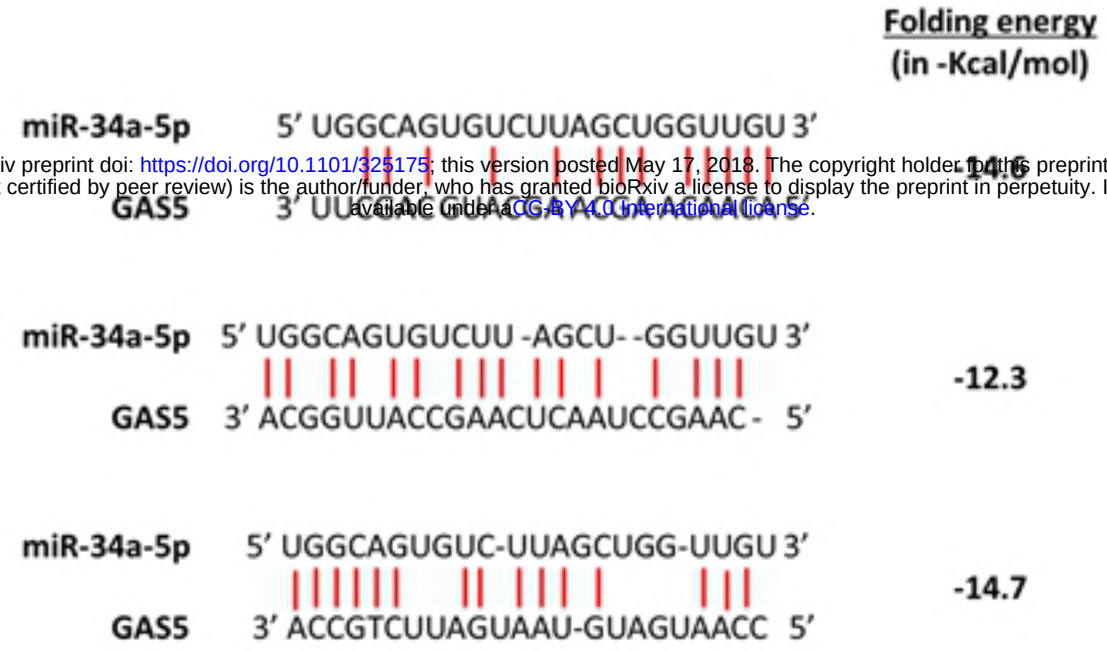

(D)



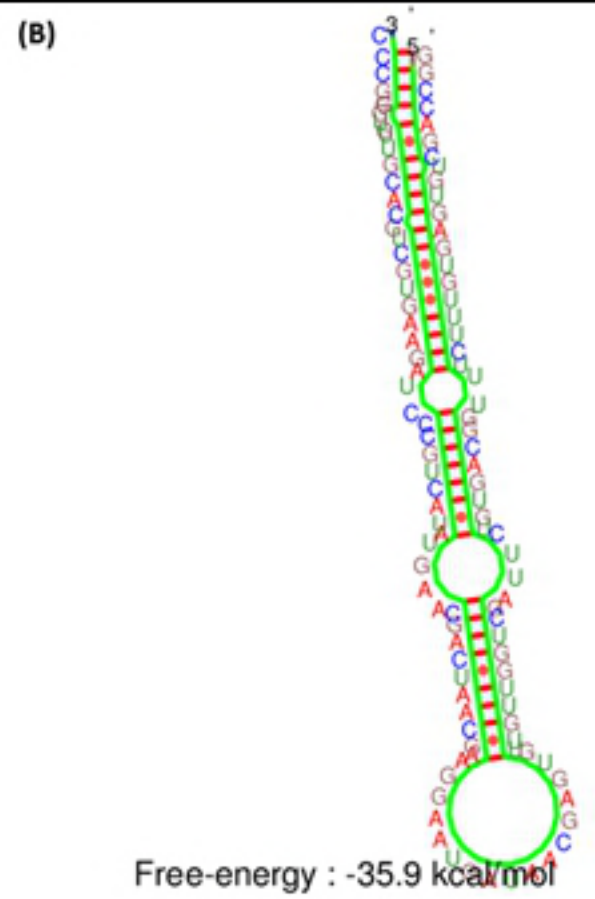

(E)



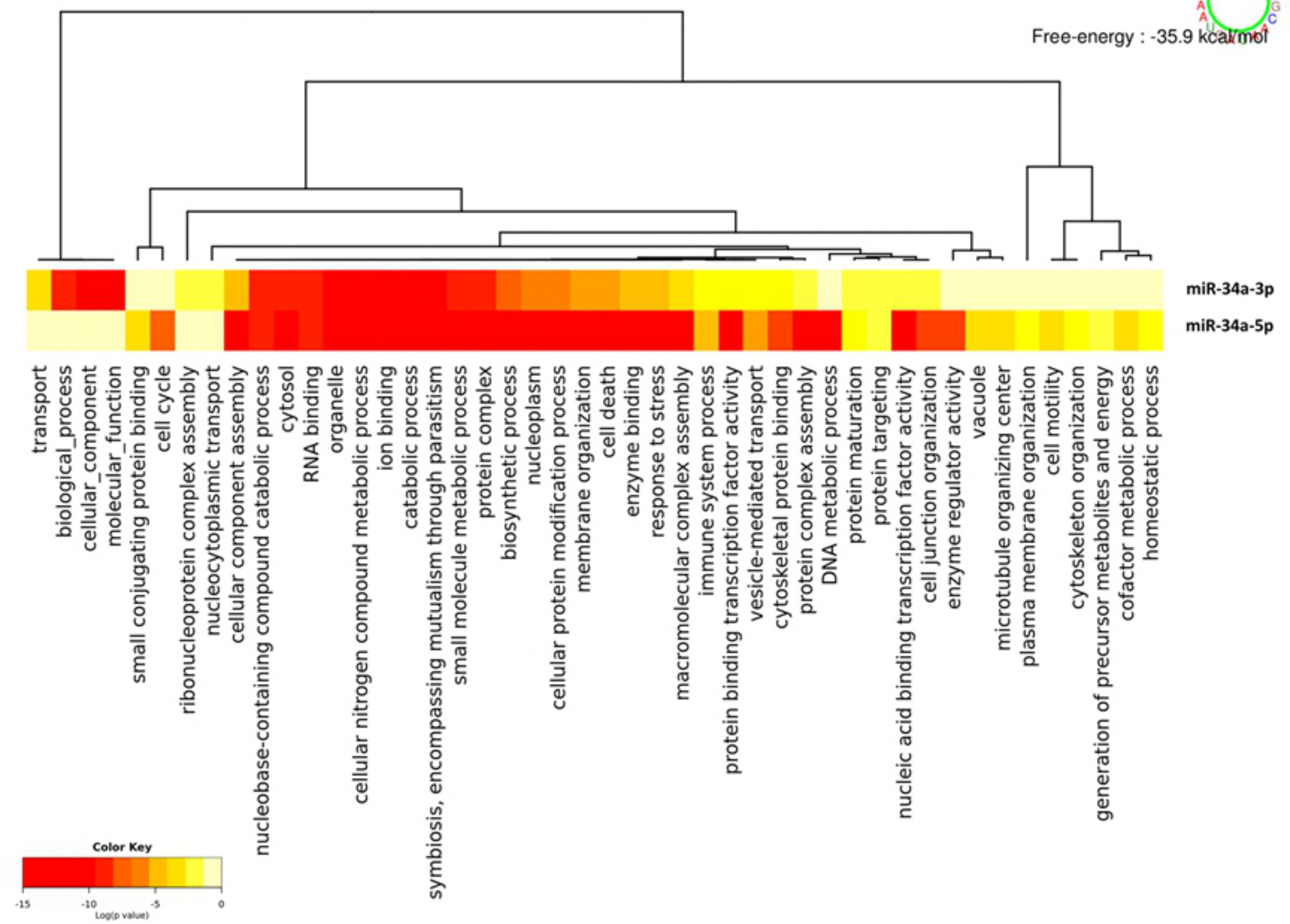

(A)

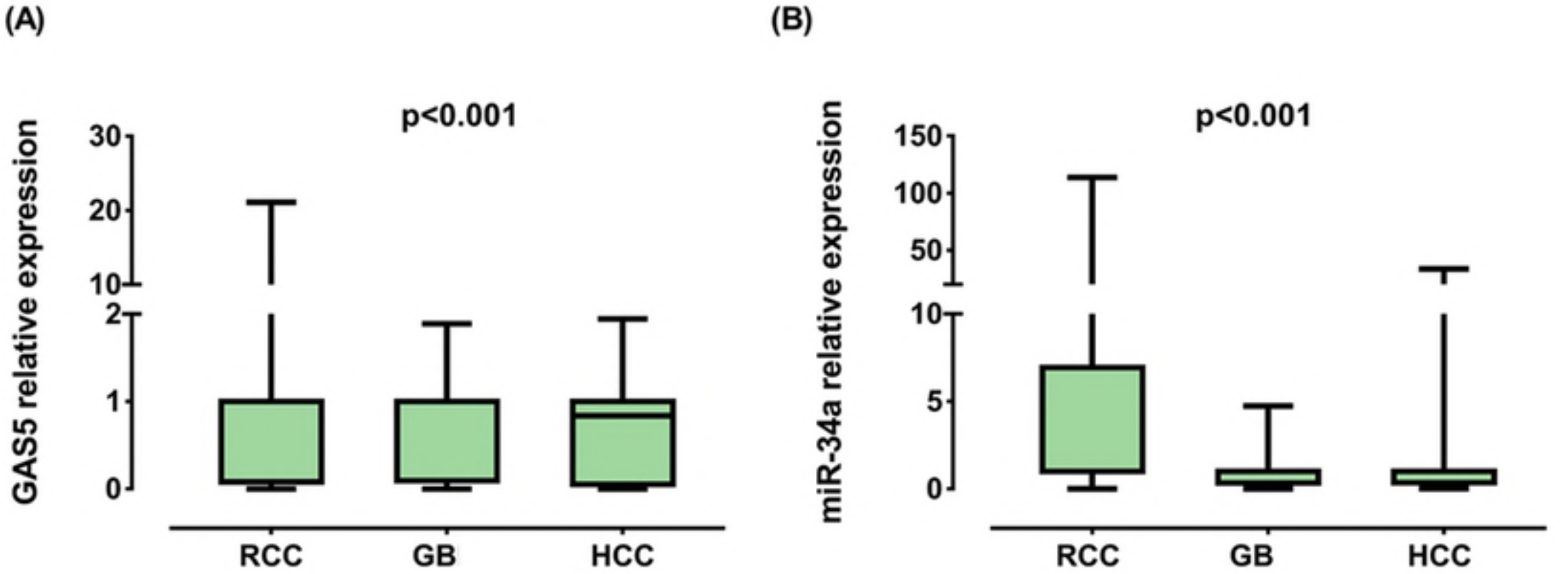


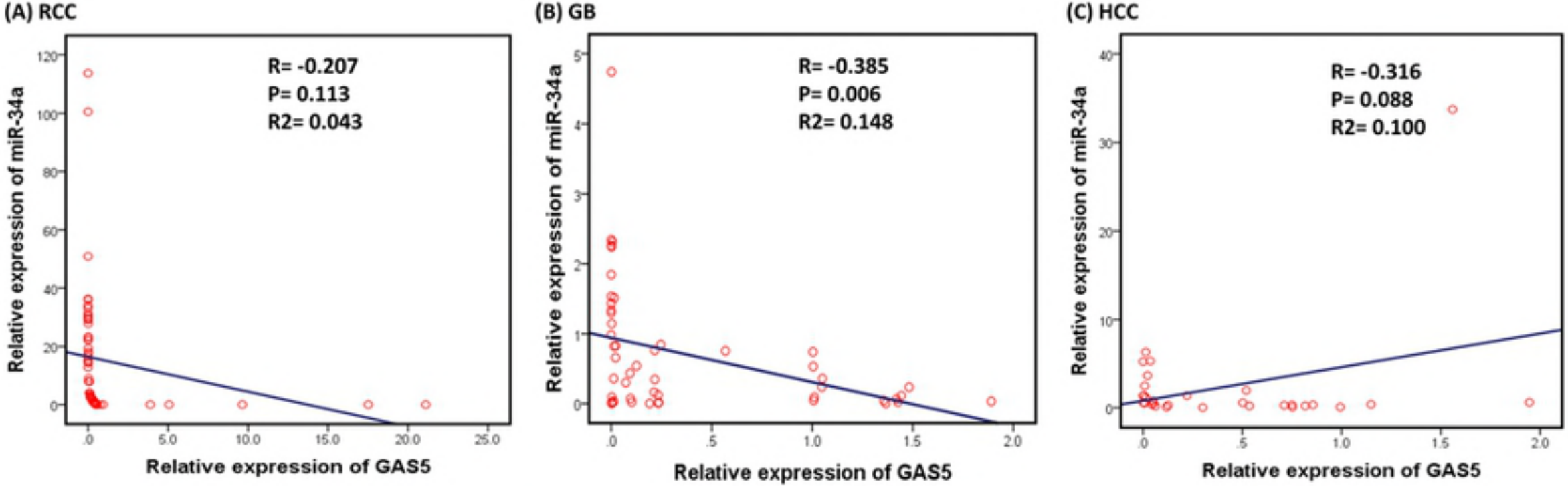

(B)

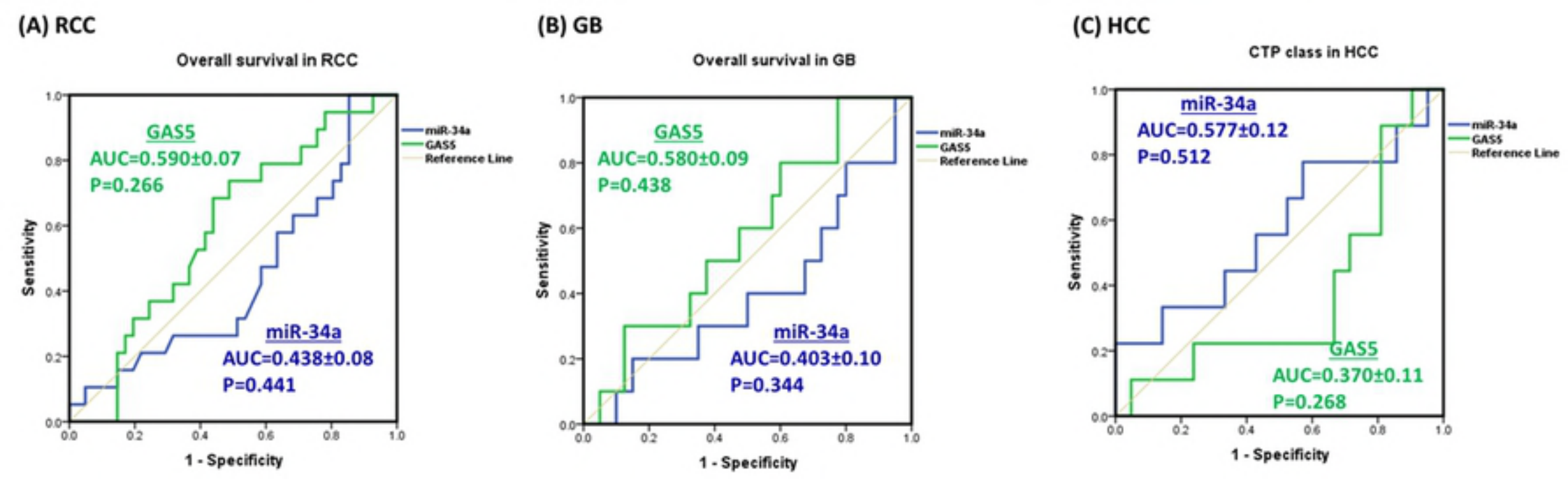


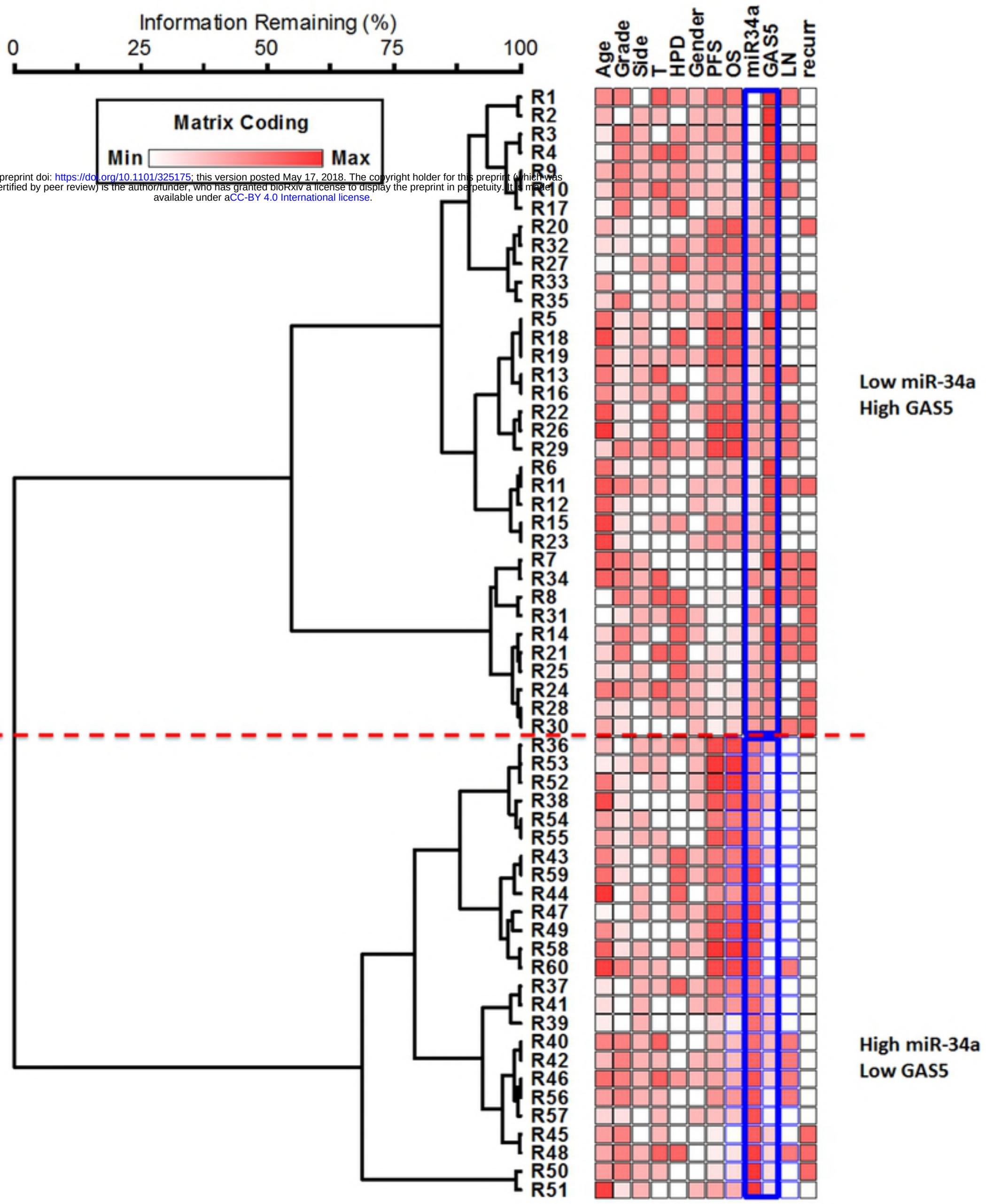

(C)

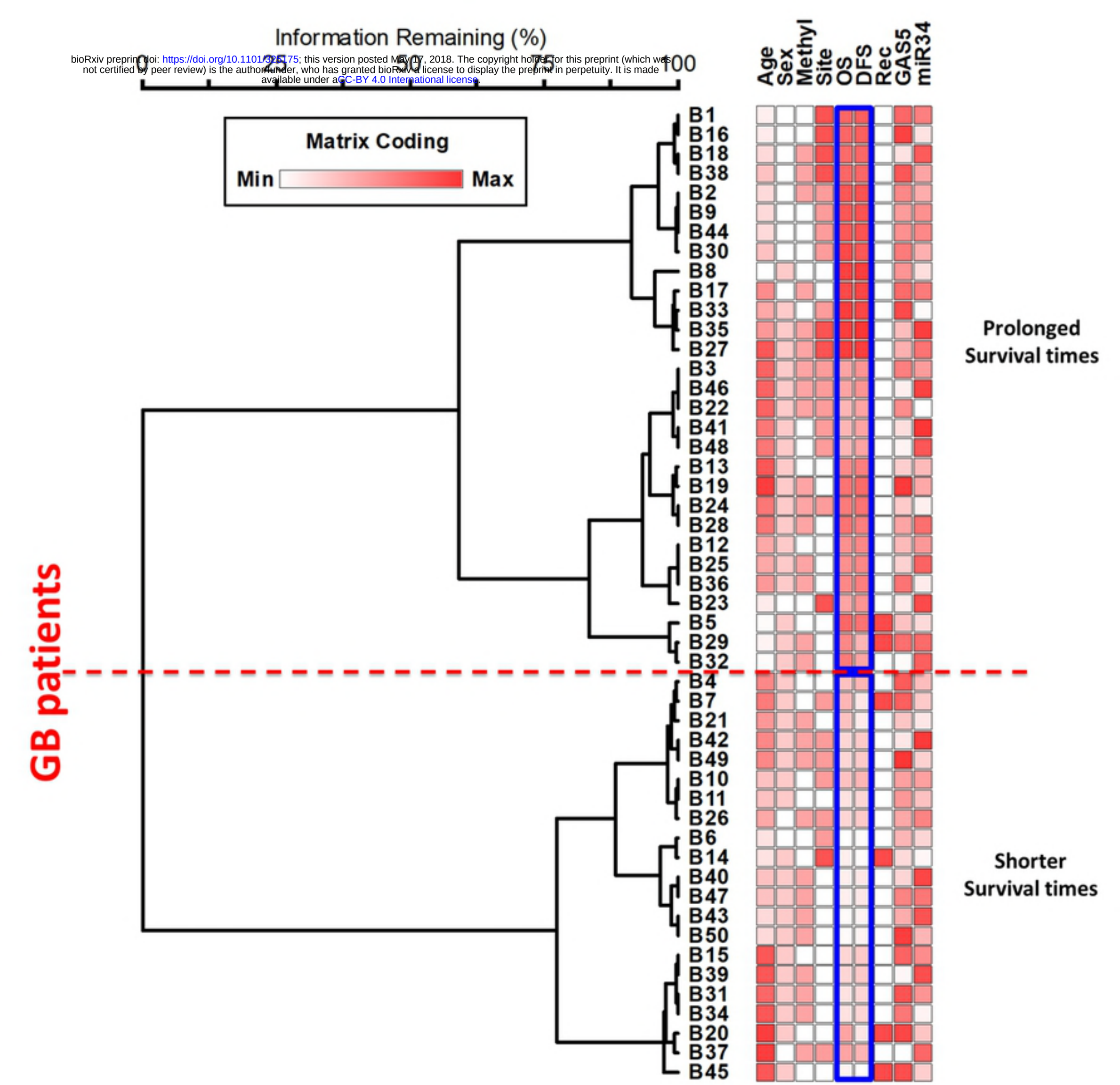


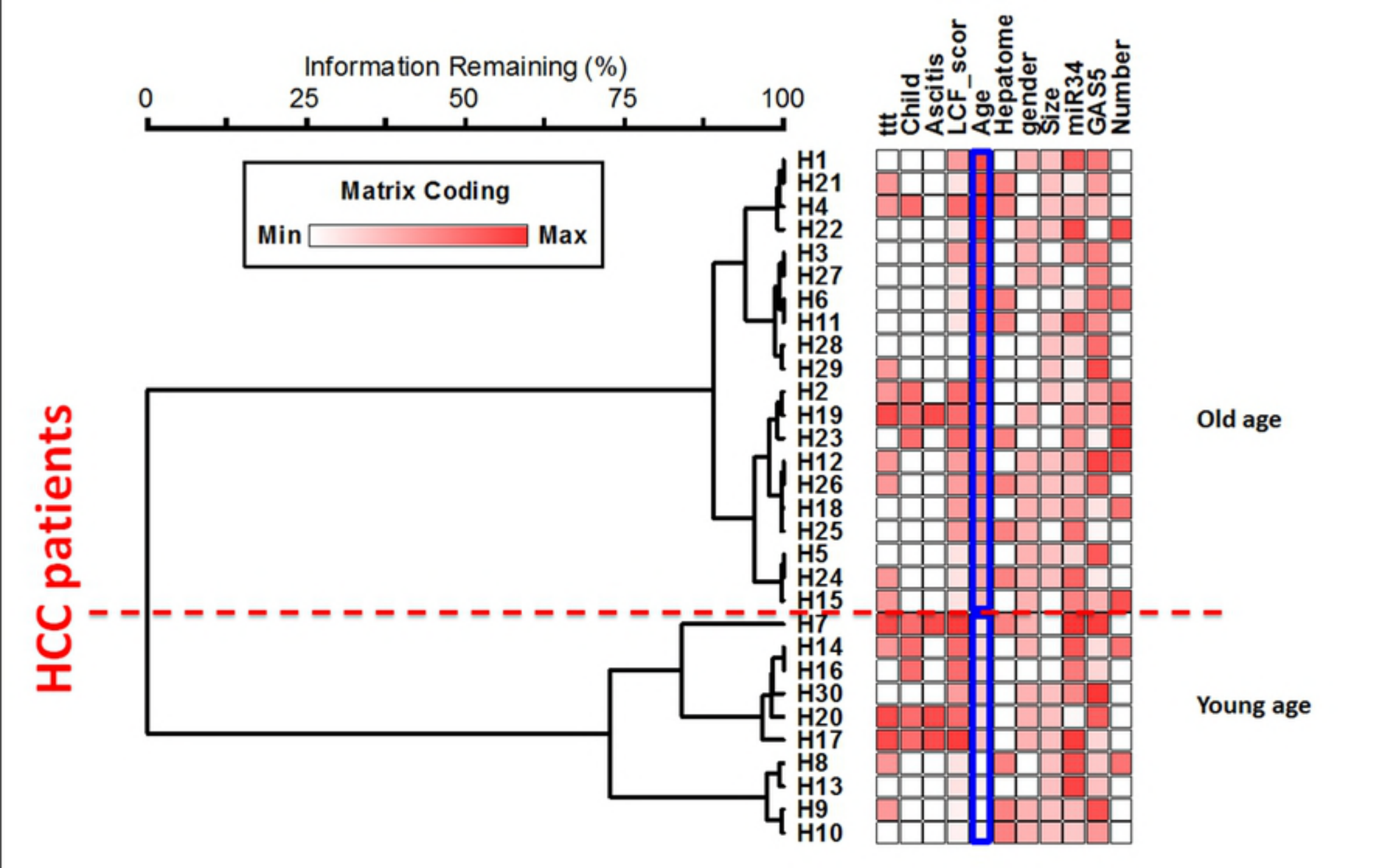

(A)
p<0.001
GAS5 relative expression
30
20
10
2
1
0
RCC
GB
HCC
(B)
p<0.001
miR-34a relative expression
150
100
50
10
5
0
RCC
GB
HCC


(A) RCC
R= -0.207
P= 0.113
R2= 0.043
Relative expression of miR-34a
Relative expression of GAS5
(B) GB
R= -0.385
P= 0.006
R2= 0.148
Relative expression of miR-34a
Relative expression of GAS5
(C) HCC
R= -0.316
P= 0.088
R2= 0.100
Relative expression of miR-34a
Relative expression of GAS5


(A) RCC
Overall survival in RCC
GAS5
AUC=0.590±0.07
P=0.266
miR-34a
AUC=0.438±0.08
P=0.441
Sensitivity
1 - Specificity
miR-34a
GAS5
Reference Line
(B) GB
Overall survival in GB
GAS5
AUC=0.580±0.09
P=0.438
miR-34a
AUC=0.403±0.10
P=0.344
Sensitivity
1 - Specificity
miR-34a
GAS5
Reference Line
(C) HCC
CTP class in HCC
miR-34a
AUC=0.577±0.12
P=0.512
GAS5
AUC=0.370±0.11
P=0.268
Sensitivity
1 - Specificity
miR-34a
GAS5
Reference Line






Information Remaining (%)
0
25
50
75
100
Matrix Coding
Min
Max
ttt
Child
Ascitis
LCF_scor
Age
Hepatome
gender
Size
miR34
GAS5
Number
H1
H21
H4
H22
H3
H27
H6
H11
H28
H29
H2
H19
H23
H12
H26
H18
H25
H5
H24
H15
H7
H14
H16
H30
H20
H17
H8
H13
H9
H10
HCC patients
Old age
Young age