Downregulated genes by silencing MYC pathway identified with RNA-SEQ analysis as potential prognostic biomarkers in gastric adenocarcinoma

Jersey Heitor da Silva Maués¹², Helem Ferreira Ribeiro¹³, Raquel de Maria Maués Sacramento², Rafael Maia de Sousa², Raissa Pereira de Tommaso², Bruno Dourado Kovacs Machado Costa², Paulo Cardoso Soares², Paulo Pimentel Assumpção⁴, Caroline de Fátima Aquino Moreira-Nunes⁵, Rommel Mário Rodriguez Burbano¹²

¹Laboratory of Human Cytogenetics, Institute of Biological Sciences, Federal University of Pará, Belém 66075-110, PA, Brazil
²Laboratory of Molecular Biology, Ophir Loyola Hospital, Belém, Belém 66063-240, PA, Brazil
³Center of Biological and Health Sciences, Department of Biomedicine, University of Amazon, Belém 66060-000, PA, Brazil
⁴Oncology Research Nucleus, University Hospital João de Barros Barreto, Federal University of Pará, Belém 66073-000, PA, Brazil
⁵Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Fortaleza 60430-275, CE, Brazil

*Equal contribution

Correspondence to: Jersey Heitor da Silva Maués; email: jerseymaues@gmail.com, https://orcid.org/0000-0001-5570-3158

Keywords: MYC, gastric carcinogenesis, distant metastases, prognosis, biomarkers

Received: June 27, 2020  Accepted: October 31, 2020  Published: December 22, 2020

Copyright: © 2020 Maués et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

MYC overexpression is a common phenomenon in gastric carcinogenesis. In this study, we identified genes differentially expressed with a downregulated profile in gastric cancer (GC) cell lines with silenced MYC. The TTLL12, CDKN3, CDC16, PTPRA, MZT2B, UBE2T genes were validated using qRT-PCR, western blot and immunohistochemistry in tissues of 213 patients with diffuse and intestinal GC. We identified high levels of TTLL12, MZT2B, CDC16, UBE2T, associated with early and advanced stages, lymph nodes, distant metastases and risk factors such as H. pylori. Our results show that in the diffuse GC the overexpression of CDC16 and UBE2T indicate markers of poor prognosis higher than TTLL12. That is, patients with overexpression of these two genes live less than patients with overexpression of TTLL12. In the intestinal GC, patients who overexpressed CDC16 had a significantly lower survival rate than patients who overexpressed MZT2B and UBE2T, indicating in our data a worse prognostic value of CDC16 compared to the other two genes. PTPRA and CDKN3 proved to be important for assessing tumor progression in the early and advanced stages. In summary, in this study, we identified diagnostic and prognostic biomarkers of GC under the control of MYC, related to the cell cycle and the neoplastic process.

INTRODUCTION

Gastric cancer (GC) is classified globally as one of the most common in the world, with high mortality and a very high incidence, mainly in East Asia, Eastern Europe, and South America [1, 2]. In 2018, data from the GLOBOCAN (Global Cancer Observatory) estimated 1,033,701 new cases of stomach cancer
worldwide, representing 5.7% of all new cancer cases. These data also estimated that gastric tumors were the third leading cause of cancer-related death in men and the fifth in women, showing that this disease is more likely to be diagnosed in men than in women [3]. GC is an aggressive disease commonly diagnosed at advanced stages, and surgical resection associated with chemotherapy or chemoradiation is considered the main treatment option [4]. The prognosis of this disease is still poor, partly as a result of local recurrence, tumor invasion, and/or metastasis [5]. The overall relative 5-year survival rate is currently less than 20% [6].

MYC dysregulation is a common event in gastric carcinogenesis, including early tumors and premalignant lesions [7]. We have shown in our studies that MYC overexpression is an important finding in Brazilian samples [8–12]. Other studies have shown that MYC amplification and overexpression was identified in 6–58% of sporadic gastric tumors [13–15], being more frequent in Brazilian samples [7, 16, 17], generally as a result of gene amplification and chromosomal translocations [5, 18]. Although studies show an association of increased MYC expression in GC, its function in gastric tumorigenesis is still unclear [19, 20] because most high-performance studies carried out so far on the genetics of GC ignore the importance of MYC in this process [5, 21–25].

In a previous study, we established and characterized three cell lines, AGP01, ACP02 and ACP03, obtained from GC with metastases of an intestinal-type, diffuse-type and intestinal-type, respectively [26]. Those cell lines also carry genetic alterations commonly found in Brazilian GC patients, such as MYC amplification and overexpression and TP53 deletion [7, 27, 28]. Also, these cell lines present the MYC silencing that was done through the interference RNA (RNAi), where we explore with bioinformatics tools the transcriptome of these three cell lines to better understand the MYC regulatory signature profile and its targets [29].

In this study, we used an analysis made on public RNA sequencing (RNA-seq) data from two cell lines mentioned above ACP02 – diffuse-type and ACP03 – intestinal-type, both silenced by the expression of MYC [29, 30]. Then, we validated the results of the transcriptome using gene expression analysis for three of the top 10 Differentially Expressed Genes (DEGs) of each cell line: TTL12, CDKN3 and CDC16 for the ACP02 and PTPRA, MZT2B and UBE2T for the ACP03 in 213 samples of gastric adenocarcinoma and their non-neoplastic pairs, as well as survival data for all patients from the time of diagnosis to the 5-year follow-up. Thus, these genes were chosen among the top 10 most differentially expressed according to the following criteria: be downregulated (regulated positively by MYC), have not yet been described in gastric cancer by our research group and are related to the cell cycle and neoplastic processes pointed out by our bioinformatics analyzes.

RESULTS

DEGs after silencing of MYC in ACP02 and ACP03 cell line

We performed an RNA-Seq data analysis from the Gene Expression Omnibus repository (GEO) with accession number GSE81265 to quantify the transcripts and their isoforms in the ACP02 and ACP03 cell lines with the silenced MYC, that reduced the expression of this gene by 84% in the ACP02 and 77% in the ACP03. Before silencing, cell phenotypes were heterogeneous, similar to a typical tumor cell line and after silencing, cells became more homogeneous, viable, but significantly lost their tumor capacity for invasion and migration as shown in our previous studies [16, 29].

From a panel of DEGs, we selected only downregulated genes with Log2 FC > 1 and p-value < 0.01. We use Log2 FC [M/C]; where M: MYC-siRNA and C: Control-siRNA. We identified 4,098 genes with a downregulated profile in ACP02 and another 842 in ACP03, to be explored in our studies, whose deregulation is directly or indirectly associated with MYC amplification, Supplementary Tables 1 and 2.

Thus, six differentially expressed genes (three from ACP02 and three from ACP03) from the top 10 (Table 1) were selected to assess their prognostic and predictive value in clinical specimens of GC tumors that exhibit MYC immunoreactivity. The following genes were selected: TTL12 (Tubulin tyrosine ligase-like family member 12), CDKN3 (Cyclin-dependent kinase inhibitor 3), CDC16 (Cell division cycle 16), PTPRA (Protein tyrosine phosphatase, receptor type, A), MZT2B (Mitotic spindle organizing protein 2B) and UBE2T (Ubiquitin-conjugating enzyme E2T).

Clinical-pathological features and expression of TTL12, CDKN3, CDC16, PTPRA, MZT2B and UBE2T in gastric cancer

We evaluated the quantitative expression of mRNA, protein and immunohistochemistry of TTL12, CDKN3, CDC16, PTPRA, MZT2B and UBE2T in 213 patient’s tumor tissues (compared to paired normal gastric tissues) with various clinical and pathological characteristics shown in Supplementary Tables 3 and 4.
TTLL12 expression was significantly high in the following variables: men under 50 years old, predominantly in tumor tissues located in the cardia, who evolved to a diffuse histological GC, with the early and non-invasive stage (T1/T2), lymph nodes negative (N0) and absence of distant metastases.

High levels of MZT2B were more significant in women over 50, identified from other parts of the stomach (antrum and body), except for cardia. These patients had a worse evolution in the clinical-pathological variables, indicating gastric cancer of the intestinal-type of serosal invasion in advanced stage (T3/T4). Positive association for lymph nodes, distant metastases and H. pylori infection.

We found high levels of CDC16 and UBE2T associated with tumor tissues with intestinal and diffuse GC, respectively. The following common scenarios were observed: predominant in women over 50 years old and with advanced serosal invasion tumors (T3/T4), strongly associated with the presence of lymph nodes and distant metastases.

CDKN3 and PTPRA were expressed in tumor tissue samples from patients of both genders. CDKN3 was most expressed in tumors of patients over 50 years old, identified mainly in the antrum and the body of the stomach. CDKN3 expression was shown to be associated with the evolution of both types of GC in patients who progressed to a positive serosal invasion of advanced stage (T3/T4), the presence of lymph nodes and distant metastases. PTPRA expression was also associated with the evolution of both types of GC in patients aged over 50 years, who have early-stage tumors (T1/T2), with this gene being more expressed in other parts of the stomach than in the cardia.

Table 1. Top-10 differential expressed genes in gastric cancer.

|Gene Symbol| Log₂FC| p-value| FDR| Gene Symbol| Log₂FC| p-value| FDR|
|-------------|--------|--------|----|-------------|--------|--------|----|
|UQCRH| -11.9 | 4.89E-38| 2.70E-37| ECT2| 12.0 | 1.47E-58| 1.35E-57|
|CDC25B| -11.6 | 6.73E-18| 1.99E-17| PSMC4| 11.9 | 2.73E-24| 9.98E-24|
|CYB561D2| -11.4 | 2.30E-51| 1.80E-50| COL8A1| 11.6 | 2.51E-31| 1.15E-30|
|TTLL12* | -11.4 | 2.56E-147| 1.06E-145| PSMD11| 11.3 | 8.94E-34| 4.41E-33|
|ARHGAPl | -11.3 | 3.00E-137| 1.10E-135| BFA1| 11.3 | 1.97E-26| 7.73E-26|
|CDKN3* | -11.2 | 3.75E-29| 1.61E-28| YWHAE| 11.3 | 3.07E-06| 5.30E-06|
|UBE2B| -10.9 | 7.22E-82| 1.08E-80| ABCF1| 11.2 | 1.97E-26| 7.72E-26|
|ATP6V0B| -10.9 | 2.09E-40| 1.23E-39| PGP| 10.9 | 1.06E-19| 3.33E-19|
|CDC16*| -10.4 | 1.38E-49| 1.03E-48| TUSC3| 10.3 | 1.22E-16| 3.42E-16|
|UFD1L| -10.7 | 1.07E-48| 7.79E-48| CKAP2L| 10.2 | 1.01E-15| 2.73E-15|

### The panel of DEGs in a gastric cell line of diffuse histological subtype
ACP02: MYC-siRNA (2M) vs. Control-siRNA (2C)

|Gene Symbol| Log₂FC| p-value| FDR| Gene Symbol| Log₂FC| p-value| FDR|
|-------------|--------|--------|----|-------------|--------|--------|----|
|PTPRA*| -12.0 | 2.33E-33| 1.56E-31| COX6A1| 12.3 | 1.37E-16| 1.91E-15|
|ATF1| -11.9 | 7.33E-30| 3.60E-28| PA2G4| 11.5 | 1.55E-49| 2.66E-47|
|UBE2T*| -11.3 | 2.02E-07| 8.82E-07| ATP6V0C| 11.4 | 4.92E-15| 5.77E-14|
|CINP| -10.9 | 2.97E-06| 1.07E-05| CIB1| 11.3 | 4.21E-14| 4.41E-13|
|TERF2| -10.8 | 1.87E-16| 2.56E-15| NDUF7A| 11.3 | 3.39E-08| 1.67E-07|
|IK| -10.7 | 2.47E-10| 1.61E-09| TNAIP3| 11.2 | 2.39E-61| 6.63E-59|
|MZT2B*| -10.7 | 4.34E-05| 0.000133| CDK2AP2| 11.2 | 9.16E-19| 1.64E-17|
|RAB23| -10.6 | 7.94E-21| 1.73E-19| COX4I1| 11.1 | 2.26E-10| 1.49E-09|
|TPDS2L2| -10.5 | 4.82E-10| 3.02E-09| CCNB1| 10.8 | 6.11E-21| 1.35E-19|
|PSMD14| -10.5 | 5.29E-08| 2.54E-07| RNF20| 10.8 | 1.00E-37| 9.01E-36|

Log₂ FC [M/C] < 0: Downregulated (left panel); Log₂ FC [M/C] > 0: Upregulated (right panel); FC: Fold change; FDR: False Discovery Rate.

Table 2. The panel of DEGs in a gastric cell line of intestinal histological subtype
ACP03: MYC-siRNA (3M) vs. Control-siRNA (3C)

|Gene Symbol| Log₂FC| p-value| FDR| Gene Symbol| Log₂FC| p-value| FDR|
|-------------|--------|--------|----|-------------|--------|--------|----|
|CDKN3*| -11.2 | 3.75E-29| 1.61E-28| YWHAE| 11.3 | 3.07E-06| 5.30E-06|
|UBE2B| -10.9 | 7.22E-82| 1.08E-80| ABCF1| 11.2 | 1.97E-26| 7.72E-26|
|ATP6V0B| -10.9 | 2.09E-40| 1.23E-39| PGP| 10.9 | 1.06E-19| 3.33E-19|
|CDC16*| -10.4 | 1.38E-49| 1.03E-48| TUSC3| 10.3 | 1.22E-16| 3.42E-16|
|UFD1L| -10.7 | 1.07E-48| 7.79E-48| CKAP2L| 10.2 | 1.01E-15| 2.73E-15|
Quantification of the gene expression in diffuse and intestinal histological types

We quantified the gene expression of the six genes mentioned above in 103 samples of GC tumor tissues of the diffuse-type and 110 of the intestinal-type. The expression data were corroborated by the analysis of protein expression. The increased levels of MYC were previously tested in our samples, according to the study by DE SOUZA et al [31]. Figure 1 shows the gene expression results of TTLL12, CDKN3, CDC16, PTPRA, MZT2B, UBE2T generated from the ACP02 and ACP03 cell lines before and after siRNA transfection, Supplementary Table 5.

TTLL12 was more expressed in tumors of diffuse GC [median mRNA (interquartile range, IQR): 1.78 (0.47); median protein (IQR): 1.70 (0.36); \( p < 0.001 \)]. In these tumor samples, there was an increase in mRNA and protein by more than 1.5-fold (at least 50% expression) estimated at 86 (83.5%) and 85 (82.5%), respectively (Supplementary Table 3).

MZT2B was most expressed in intestinal GC tumor samples [mRNA median (IQR): 1.43 (0.51); protein median (IQR): 1.52 (0.45); \( p < 0.001 \)]. Also, mRNA levels reached 48 (43.6%) and the protein increased more than 1.5-fold in 57 (51.8%) in these tumor samples (Supplementary Table 4).

We did not find significant differences mRNA and protein expression levels of CDKN3, PTPRA and UBE2T between the two histological types (Supplementary Tables 3 and 4). For CDC16, we found only protein levels with a significant increase in intestinal tumor samples (\( p = 0.015 \)). In these tumor samples, we find the

Figure 1. Effect of MYC silencing on gene and protein expression in ACP02 and ACP03. The heat maps with hierarchical grouping show how the levels of gene and protein expression of TTLL12, CDKN3, CDC16, PTPRA, MZT2B, UBE2T are related to MYC in the ACP02 (A) and (B) and ACP03 (C) and (D). All comparisons were made concerning non-neoplastic gastric mucosa MNP01. Z-score was the metric applied to test the clustering between genes. Blue gradients represent a lower Z-score (genes with a lower level of expression) and red gradients represent a higher Z-score (genes with a higher level of expression).
following measures of gene expression [mRNA median (IQR): 1.66 (0.51); protein median (IQR): 1.50 (0.63)] (Supplementary Table 3). mRNA and protein levels increased more than 1.5-fold in 77 (70.0%) and 58 (52.7%), respectively. Our results of gene expression are summarized in the graphs of Figure 2.

Corroborating the results of qPCR, western blot and immunoreactivity, it can reveal significant differences of expression in samples with different TNM and in different histological types (Figures 3A and 3B–3G). Also, our protein expression results for the DEGs of the ACP02 and ACP03 were shown to be correlated with those of The Human Protein Atlas, revealing significant expression profiles in the stomach cells (Figure 3H–3I). Relationship between the expression of TTLL12, MZT2B, CDC16 and UBE2T with metastatic progression.

Gene expression results from Supplementary Tables 3 and 4 was used to establish an association between the increase in mRNA and protein of the TTLL12, MZT2B, CDC16 and UBE2T genes with a possible metastatic progression of patients M0 and M1. We identified increased levels of TTLL12, CDC16 and UBE2T in tumor samples from 103 patients with diffuse GC (M1) (p <0.001 for all analyzes; Figure 4A and 4B).

Likewise, we identified an increase in mRNA and protein levels in tumor samples from 110 patients with intestinal GC (M1), for MZT2B and CDC16 (p <0.001 for all analyzes; Figure 4C and 4D). These results are corroborated when we evaluate the increased levels of mRNA and protein in tumor samples from patients M1 that were analyzed by histological types (Figure 4E and 4F). These results differ from those found in Supplementary Tables 3 and 4 because it takes into account the total samples (n = 213).

Association of the expression profile with survival

Our results of TTLL12, MZT2B, CDC16 and UBE2T expressions showed that higher levels of TTLL12 are associated to early-stage tumors (T1/T2) in the diffuse-type (Figure 5A), in early-stage tumors (T1/T2) (Figure 5A). We identified higher levels of CDC16 and UBE2T expression in diffuse advanced-stage GC tumors (T3/T4) (Figure 5B, 5C). InGC tumors

![Figure 2](image-url)

**Figure 2.** Level of mRNA and protein expression of TTLL12 (A), CDKN3 (B), CDC16 (C), PTPRA (D), MZT2B (E), and UBE2T (F) in diffuse (n = 103) and intestinal (n = 110) GC tumor samples. Mann–Whitney test was used to compare the relative gene expression levels. In all graphs, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. RQ: relative quantification; T: tumor sample; N: normal mucosa sample; The whiskers indicate the minimum and maximum values. red dotted line represents the 1.5 fold-change.
Figure 3. Analysis of protein expression and immunohistochemistry in gastric cancer subtypes. (A) Representative image of Western-blot. Line 1 represents normal gastric tissue and the intensity of the genes are similar to early TNM stages and without metastasis, but much lower than advanced TNM stages (T3/T4). Initial stages with metastasis also have an intensity much higher than that of normal gastric tissue. (B) Positive TTLL12 cytoplasmatic immunostaining in diffuse-type gastric cancer (case 66 T2N3M1); (C) Positive CDC16 cytoplasmic and nuclear immunostaining in diffuse-type gastric cancer (case 140 T3N3M0); (D) Positive CDKN3 cytoplasmatic immunostaining in diffuse-type gastric cancer (case 203 T4N2M1); (E) Positive PTPRA cytoplasmatic immunostaining in intestinal-type gastric cancer (case 5 T1N0M0); (F) Positive MZT2B cytoplasmatic immunostaining in intestinal-type gastric cancer (case 61 T2N3M0); (G) Positive UBE2T cytoplasmatic and nuclear immunostaining in intestinal-type gastric cancer (case 149 T3N3M1) (magnification x40). The differences in band intensity and intensity of immunoreactivity are due to the different stages of TNM in tumor samples of diffuse and intestinal histological types that represent figures (3A, 3B–3G). (H and I) The function of the DEGs in the gastric lines showed a strong correlation between the increased level of protein expression with the human stomach cells in the data from The Human Protein Atlas (HPA) that were accessed and normalized in -log10 p-value (adj.). The levels of protein expression of the DEGs are identified by the red rectangle in 15 subtypes of human stomach cells.
of the intestinal-type, the expressions of \textit{MZT2B}, \textit{CDC16} and \textit{UBE2T} were higher only in advanced-stage tumors (T3/T4) (Figure 6A–6C).

Analysis of the area under the ROC curve (AUC) to classify patients with high and low gene expression associated with survival capacity showed a cutoff point as the highest point of the AUC for the \textit{CDC16}: AUC = 0.812, \( p < 0.0001 \); followed by \textit{TTLL12} and \textit{UBE2T} that exhibited practically the same area under the curve with AUC = 0.713, \( p < 0.0001 \) and AUC = 0.715, \( p < 0.0001 \), respectively (Figure 5D). We identified the cut-off

---

**Figure 4.** Box plot shows the relationship between the normalized expression of \textit{TTLL12}, \textit{MZT2B}, \textit{CDC16} and \textit{UBE2T} with metastatic progression. (A and B) mRNA and protein levels of \textit{TTLL12}, \textit{CDC16} and \textit{UBE2T} in 103 tissue samples with diffuse-type GC from patients \( M_0 \) and \( M_1 \). (C and D) mRNA and protein of \textit{MZT2B}, \textit{CDC16} and \textit{UBE2T} in 110 tissue samples with intestinal-type GC from patients \( M_0 \) and \( M_1 \). The boxes are drawn from the 75th to the 25th percentile. The vertical lines above and below the box define the maximum and minimum values and the dots indicate outliers, the horizontal line inside the box representing the median. (E and F) mRNA and protein of \textit{TTLL12}, \textit{MZT2B}, \textit{CDC16} and \textit{UBE2T} in patients with diffuse (n = 103) and intestinal (n = 110) GC that were associated with \( M_0 \) and \( M_1 \). The lines with the gray gradient represent the connections of points \( M_0 \) and \( M_1 \) that vary according to the normalized expression value. The expression levels of these genes were validated by qRT-PCR and western blot in 213 patients. (M) presence of metastasis. Mann–Whitney test was used to compare the relative gene expression levels.
Figure 5. Quantification of TTLL12 (A), CDC16 (B) and UBE2T (C) mRNA and protein levels by tumor stage in GC of the diffuse-type. *p <0.008, **p <0.001, ***p <0.0002, ****p <0.0001, significant difference between groups (Nonparametric Mann–Whitney test). In all graphs, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. RQ: relative quantification; T: tumor sample; N: normal mucosa sample. (D) ROC curve analysis. The cutoff point was chosen as the highest point of the Area-Under-the-Curve (AUC) for TTLL12, AUC: 0.713; (95% CI 0.62-0.76) with 76.02% sensitivity and 50.04% specificity, for CDC16, AUC: 0.812; (95% CI 0.70-0.83) with 85.0% sensitivity and 50.08% specificity and for UBE2T, AUC: 0.715; (95% CI 0.63-0.77) with 77.06% sensitivity and 48.03% specificity. (E) Heat maps showing the average levels of gene expression of ~35 samples of GC tissues of the diffuse-type. The red gradient shows the highest levels of expression while the blue gradient shows the lowest levels. Kaplan-Meier analysis of the overall survival (in months) of patients with diffuse gastric cancer as a function of (F) TTLL12, (G) CDC16 and (H) UBE2T expression. We analyzed high expression (gene expression ≥1.5; red line), as opposed to low expression (gene expression <1.5; blue line) associated with a higher probability of survival. We found more significant values when we associated the high expression of CDC16 (p <0.0001) and UBE2T (p = 0.0024), with patients who had a shorter survival.
values of 1.65 for CDC16, 1.64 for TLL12 and 1.56 for UBE2T. The same previous analysis indicated for MZT2B: AUC = 0.803, p < 0.0001; CDC16: AUC = 0.798, p < 0.0001; UBE2T: AUC = 0.549, p < 0.01. From these data, we identified cut-off values of 1.45 for MZT2B, 1.65 for CDC16 and 1.56 for UBE2T (Figure 6D). The heat maps show the levels of gene expression in ~ 35 samples of GC tissues from patients M0 and M1 of the diffuse and ~ 30 of the intestinal, confirming how the gene expression increases according to the tumor stage (Figures 5E and 6E).

In the first year after the diagnosis of diffuse GC, patients with high expression of TLL12 (Log-rank test, p = 0.12) showed no association with decreased survival and their overexpression is not related to poor prognosis, as patients with higher levels of TLL12 exhibited increased survival. On the other hand, the Kaplan-Meier analysis demonstrated an association between the high expression of the CDC16 (Log-rank test, p < 0.0001) and UBE2T (Log-rank test, p = 0.0024) with decreased overall survival patients, indicating a worse prognosis (Figure 5F–5H).

In the first year after the diagnosis of intestinal GC, the Kaplan-Meier analysis showed a significant result only for the association between the high expression of CDC16 (Log-rank test, p = 0.0085) with the decrease in the overall survival of the patients and consequently, worse prognosis. Patients with high expression of MZT2B (Log-rank test, p = 0.57) and UBE2T (Log-rank test, p = 0.100) did not show a significant association with decreased survival (Figure 6F–6H).

**Gene set enrichment analysis (GSEA) in ACP02 and ACP03**

We identified increased and decreased pathways in both gastric cells line, based on hallmark gene sets (“Hallmark gene sets summarize and represent specific well-defined biological states or processes and display coherent expression”, as defined by GSEA), KEGG and gene ontology (Figure 7).

In the ACP02 we identified a decrease in the expression of genes related to the hallmark gene set that was more significant for mTORC1 signaling, MYC targets and Oxidative phosphorylation, while in the ACP03 we identified an increase in the expression of more significant genes for Adipogenesis, E2F targets, G2M checkpoint, mitotic spindle, oxidative phosphorylation and protein secretion (Figure 7A, 7B).

We still found a wide repertoire of KEGG metabolic pathways inhibited in ACP02, mainly for cellular senescence, ribosome, ubiquitin-mediated proteolysis (Figure 7C). The analysis of the ontology gene revealed many genes of this lineage related to a mitochondrial cell component, playing a role in the metabolic process of lipids, phospholipids, amino acids and RNA catalytic activities, in addition to several molecular functions related to small GTPases (Figure 7E).

The main pathways activated in ACP03 were for ubiquitin-mediated proteolysis, oxidative phosphorylation, cell cycle (Figure 7D). Most of the genes activated in these cells were noted, mainly, in nuclear components and organelles such as the Golgi apparatus and mitochondria, exercising functions in protein catalysis, signal transduction and cell cycle (Figure 7F). The total result of this analysis can be accessed in Supplementary Table 6.

**DISCUSSION**

MYC has a fundamental role in gastric carcinogenesis, being one of the most robust and significant prognostic markers in the GC [32]. MYC amplification and its upregulated expression were commonly observed in cell lines and tissues of GC. The highest MYC levels have been reported in the tumors of patients with local or distant metastases [7, 8, 11, 12, 28, 31–36]. It is therefore critical to understand how the MYC knockdown alters the expression of several genes involved in gastric carcinogenesis, being an important step in the discovery of new targets activated by this gene [37, 38].

In this work, qPCR and western blot results revealed a significant increase in expression levels of TLL12, MZT2B, CDC16 and UBE2T in GC tumor samples of diffuse and intestinal-type, when compared with normal gastric tissues. We also evaluated the levels of CDKN3 and PTPRA that were not efficient to differentiate the two histological types of the disease. They are useful only for identifying the GC.

The expression of TLL12 was more significant in the GC of the diffuse-type located in the cardia region of male patients under 50 years of age, who had the disease at an early-stage and a longer survival during the first year after diagnosis, as found in other studies [39, 40]. Such characteristics added to a high prevalence of this neoplasia in the north and northeast of Brazil [41], reinforces the importance of an early screening method for this type of GC. This gene can be used as a biomarker to identify diffuse GC early, as we have identified increased levels of TLL12 in tumor samples from patients predisposed to metastatic progression. TLL12 is a gene capable of modifying tubulins, a marker that can be studied and deepened in the progression and metastasis of GC. Studies show that the levels of the c-MYC oncoprotein activated in vitro and
in vivo conditions exert its effects on alpha-tubulin and polymerized microtubules [42, 43]. TTLL12 is reported to be abnormal in many cancer cells [44–46], but its function in GC is still unknown.

The high expression of MZT2B was more significant in GC samples of the intestinal histological type, identified exclusively in the antrum and the stomach body [47] of female patients over 50 years of age. These patients had

**Figure 6.** Quantification of MZT2B (A), CDC16 (B) and UBE2T (C) mRNA and protein levels by tumor stage in GC of the diffuse intestinal. *p <0.008, **p <0.001, ***p <0.0002, ****p <0.0001 significant difference between groups (Nonparametric Mann–Whitney test). In all graphs, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. RQ: relative quantification; T: tumor sample; N: normal mucosa sample. (D) ROC curve analysis. The cutoff point was chosen as the highest point of the Area-Under-the-Curve (AUC) for MZT2B, AUC: 0.803; (95% CI 0.69–0.82) with 84.2% sensitivity and 50.0% specificity, for CDC16, AUC: 0.798; (95% CI 0.69–0.82) with 86.0% sensitivity and 49.08% specificity and for UBE2T, AUC: 0.549; (95% CI 0.51–0.68) with 52.03% sensitivity and 47.05% specificity. (E) Heat maps showing the average levels of gene expression of ~30 samples of GC tissues of the intestinal-type. The red gradient shows the highest levels of expression while the blue gradient shows the lowest levels. Kaplan-Meier analysis of the overall survival (in months) of patients with gastric cancer of the intestinal-type as a function of the expression of (F) MZT2B, (G) CDC16 and (H) UBE2T. We analyzed high expression (gene expression ≥1.45; red line), as opposed to low expression (gene expression <1.45; blue line) associated with a higher probability of survival. We found more significant values only for high expression of CDC16 (p <0.0085) when associated with the lower shortest patient survival.
worse clinical and pathological evolution, developing distant metastases, infection by *H. pylori* and shorter survival. To date, only one study has identified MZT2B as a target for MYC in gene expression data for breast cancer cell lines and tissues [48]. MZT2B is an important binding protein in the organization of the mitotic spindle. We suggest that the elevated expression of MZT2B is activated by MYC in gastric carcinogenesis resulting in dramatic changes in the extracellular matrix and the cell communication pathways [49] associated with tumor progression, intestinal metaplasia and exposure to risk factors such as *H. pylori* infection [50].

*CDC16* and *UBE2T* showed high levels in the intestinal and diffuse GC, capable of compromising the antrum

**Figure 7.** GSEA analysis of the DEGs in ACP02 and ACP03. (A and B) GSEA Enrichment plot of the main sets of genes enriched in the hallmark collection in both cell lines. Three major important KEGG pathways were inhibited in ACP02 (C) and activated in ACP03 (D). GO (Gene ontology) analysis was identified in the transcriptomes in ACP02 (E) and ACP03 (F) for the categories of cellular component, biological process, and molecular function. In the hallmark and KEGG charts, the enrichment score values assume negative values (rank of inhibited genes) for ACP02 and positive values (rank for activated genes) for ACP03, as indicated in the curves. The FDR is the estimated probability that a gene set with a given NES (normalized enrichment score) represents a false-positive finding. Several enriched gene sets are significant as indicated by an FDR of less than 25%. In all analyzes *p*-value <5%.
region and the stomach body, especially in women over 50 years old. The increased expression of these two genes in the diffuse GC is associated with decreased survival, related to the variables of worse prognosis, which was not observed in the survival results of patients who expressed UBE2T in the intestinal-type. MYC can activate the protein encoded by CDC16 which functions as an anaphase promoter complex, mediated by a ubiquitin ligase that activates the progression of mitosis and the G1 phase of the cell cycle, controlling the onset of DNA replication [51].

Studies with CDC16 in the GC are still scarce, but the involvement of this gene has been reported in metastasis of prostate [52], lung [53] and human colon [54]. The function of UBE2T has been reported in the progression of GC as a potential prognostic biomarker [55]. The ubiquitin-conjugating enzyme E2T (UBE2T) is a member of the E2 family that mediates the ubiquitin-proteasome system that acts on gene expression [55, 56]. UBE2T activates cell mobility, inducing the fundamental epithelial-mesenchymal transition in gastric tumorigenesis.

The expression levels of CDKN3 and PTPRA were quantified without significant differences between the two histological types, representing two important biomarkers to detect GC without differentiating the disease. CDKN3 was more associated with the worst prognosis variables in the advanced GC than PTPRA, which was more associated with the variables with a better prognosis. CDKN3 cyclin-dependent kinase inhibitor 3, is a cyclin-dependent kinase (CDKs) that controls cell cycle progression and other critical functions within the cell [57]. CDKN3 can be recruited by MYC to keep many mitotic cells responsible for promoting tumorigenesis [58]. MYC can activate tyrosine phosphatase signaling pathways which are important mechanisms involved in signal transduction in cancers [59]. Possibly, MYC increases the levels of PTPRA, activating the function of this gene as a tumor suppressor, enabling cell signaling events, coordinating the control of proliferation, apoptosis, survival, migration and invasion [60, 61].

Our results of functional annotation of the DEGs of the gastric cell lines revealed a strong correlation between the increase in protein expression with the stomach cells. Confirming the effects of MYC on the gene expression of these cell lines. We still found strong evidence that the silencing of MYC in both cells’ lines, activated and inhibited some sets of genes and important carcinogenesis pathways with a direct impact on cell senescence, ribosome assembly, ubiquitin-mediated by proteolysis, oxidative phosphorylation, cell cycle control, strongly associated with TTLL12, CDKN3, CDC16, PTPRA, MZT2B and UBE2T.

The identification of new diagnostic biomarkers and prognoses of the GC is urgently needed. Approaches involving integrated bioinformatics for mining transcriptomic, epigenomic and other types of broad genome associations are effective tools for identifying new biomarkers associated with pathways and modules related to disease subtypes [62–64]. We suggest that only the analysis of MYC-activated genes in cell lines is not entirely conclusive. The most important information comes from validating data collected from clinical samples of tumors and surrounding normal tissues. The genes identified in this study proved to be promising biomarkers for the identification of GC and have been validated in a considerable number of samples (210). However, to consolidate their clinical applicability, we strongly recommend that they be included in evaluations on large scales of patients (above 1000).

**CONCLUSION**

In summary, our results showed that in the diffuse GC, overexpression of CDC16 and UBE2T indicate markers of poor prognosis higher than TTLL12, that is, patients with overexpression of these two genes live less than patients with overexpression of TTLL12. In the intestinal GC, patients who overexpressed CDC16 had significantly shorter survival than patients who overexpressed MZT2B and UBE2T, confirming the worse prognostic value of CDC16 in this disease. We also emphasize the importance of PTPRA and CDKN3, which are promising to assess tumor progression in early and advanced stages of the GC, without differentiating the histological types.

**MATERIALS AND METHODS**

**Cell lines used in this study**

In this study, ACP02 and ACP03 cell lines that were obtained from diffuse and intestinal GC, respectively established and previously characterized by our group, were used [26]. Cell culture of non-neoplastic gastric mucosa cells (Normal Gastric Mucosal Cell Line 01, MNP01) was also used to initially evaluate the gene and protein expression of TTLL12, CDKN3, CDC16, PTPRA, MZT2B and UBE2T. The ACP02, ACP03 and MNP01 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Invitrogen, Germany) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were maintained in a 5% CO₂ air-humidified atmosphere at 37° C [16].
MYC-silencing in ACP02 and ACP03

A total of 2x10^3 ACP02 and ACP03 cells were seeded in 12-well culture plates after 24 h MYC silencing was done by transfecting ACP02 and ACP03 cells with a solution of Lipofectamine® RNAiMAX Transfect Reagent (Life Technologies, USA) diluted in Opti - MEM® (Life Technologies, USA) mixed with the control siRNA or the target gene-targeted siRNA. SiRNAs used against the MYC gene constituted a pool of four different commercially obtained previously validated synthetic siRNAs (SMARTpool ON-TARGETplus MYC siRNA L-003282-02-0020, Dharmacon, GE Lifesciences, USA) at a concentration of 20 µM; The negative control was a 4 siRNA pool whose sequence was not the target of any known transcript (ON-TARGETplus Non-targeting Pool D-001810-10-05, Dharmacon, GE Lifesciences, USA).

For MYC silencing, a pool of four different double-stranded siRNAs targeting MYC (20 µM; SMARTpool ON-TARGETplus MYC siRNA, L-003282-02-0020; GE Healthcare Dharmacon, USA) or scrambled control siRNAs (ON-TARGETplus Nontargeting Pool, D-001810-10-05; GE Healthcare Dharmacon, USA) were transfected into ACP02 and ACP03 cell lines using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, USA). All siRNA experiments were performed three times.

Data analysis

In this study, we used public RNA-Seq data that was obtained with Ion Proton™ from two gastric cancer cell lines ACP02 and ACP03. This data is deposited in the Gene Expression Omnibus (GEO) under the access number GSE81265, which was previously analyzed [29]. In our analysis, ACP02 cell line control was named 2C, and its MYC-silenced counterpart was named 2M. Similarly, ACP03 cell line control was termed as 3C and the MYC-silenced was named 3M.

Identification and analysis of DEGs

Differential Expressed Genes (DEGs) were identified between MYC-siRNA (M) and control-siRNA (C) from ACP02 and ACP03 lines. We use the following comparisons in the ACP02 cell line (2M versus 2C) and the ACP03 cell line (3M versus 3C). To identify the DEGs between two paired samples, we used the Audic-Claverie test [65]. DEGs with a cutoff point of \[ |\log_2 \text{Fold change (FC)} | > 1 \] and \( p\)-value <0.01, FDR <0.01 were calculated as the \( \log_2 \) ratio between the silenced and the control sample. Among the top-10 downregulated genes evaluated in the silenced MYC cell lines, six genes were selected (TTLL12, CDKN3, CDC16, PTPRA, MZT2B and UBE2T) and validated by qPCR, western blot and immunohistochemistry in samples of tumor tissues of patients with GC. Such genes were selected because we consulted several studies in the literature reporting their involvement with other types of cancer, but we did not find a direct implication with the GC.

Functional analysis of DEGs in gastric cancer

The functional analysis of the DEGs was done in two moments. First, we used the g:Profiler [66] tool to investigate the correlation between the protein expression of the ACP02 and ACP03 DEGs with stomach cells using The Human Protein Atlas (https://www.proteinatlas.org/). Protein expression data were normalized on the -log_{10} p-value scale (adj.), which showed significance for 15 different cell types of the human stomach.

Then, the DEGs of the ACP02 and ACP03 lines were analyzed separately with the fgsea package [67]. Complementary analyzes were done with ClusterProfiler packages [68]. We used three collections of gene expression data from The Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb), Hallmark gene set, Gene Ontology and KEGG: Kyoto Encyclopedia of Genes and Genomes. Permutation was conducted 1,000 times according to default-weighted enrichment statistics and by using a Signal2Noise metric to rank genes. Set genes with 10 to 500 genes and FDR <25% were selected for evaluation. Significant gene sets were defined as those with a nominal \( p\)-value <0.05.

Patients and tissue specimens

A total of 213 patients’ samples of gastric adenocarcinoma and its non-neoplastic counterparts were obtained with local Ethics Committee approval number: 2.340.667 from the Ophir Loyola Hospital, Belém, Brazil. Signed informed consent was obtained from all patients before sample collection. All methods were carried out following the relevant guidelines and regulations. All samples were classified according to Lauren and the tumors were staged according to the tumor-node-metastasis (TNM) staging criteria [69]. The presence of Helicobacter pylori (H. pylori) in gastric samples was detected by the rapid urease test, and its virulence factor cytotoxicity-associated gene A (CagA gene) was detected by polymerase chain reaction (PCR) using DNA purified simultaneously with proteins and mRNA, as previously performed by our group [70]. Epstein-Barr virus (EBV) was detected by RNA in situ hybridization.
mRNA expression

After the total mRNA was isolated, it was reverse-transcribed using the High-Capacity cDNA kit according to the manufacturer’s protocol (Thermo Fisher Scientific, USA). The mRNA and cDNA concentration and quality were determined using a NanoDrop spectrophotometer (Kisker, Germany) and 1% agarose gels, respectively. Samples were stored at -80 °C until use. cDNA was then amplified by real-time quantitative PCR (qPCR) using TaqMan probes: TTI12: Hs00209450_m1, CDKN3: Hs00193192_m1, CDC16: Hs00187430_m1, PTPRA: Hs00160751_m1, MZT2B: Hs01117110_sH, UBE2T: Hs00928040_m1 and ACTB (4333762F; Thermo Fisher Scientific, USA) gene was selected as an internal control [27]. All qPCRs were performed in triplicate in 7500 Fast Real-Time PCR instrument (Thermo Fisher Scientific, USA).

The relative quantification of gene expression was calculated according to the method of Livak and Schmittgen [71]. We used a control sample of non-neoplastic gastric mucosa cells MNP01 (Normal gastric mucosa cell Line 01) pooled from 10 healthy patients [16], which was used as a calibrator for each tumoral sample. The mRNA and protein data are expressed as the median and interquartile range (IQR) of fold change in gene expression level in the gastric tumors normalized to the ACTB gene and relative to levels in the adjacent non-neoplastic control sample.

Protein quantification

Western blot analysis was performed as described previously [16]. Reduced protein (25 μg) from each sample was applied to SDS-polyacrylamide gel and electrophoresed. Then the individual proteins in the electrophoresis gel were transferred to a polyvinylidene fluoride membrane and labeled with antibodies specific for the corresponding DEG proteins: anti-TTI12 (dilution 1:2000; MA5-25018; Thermo Fisher Scientific, USA) anti-CDKN3 (1:2000 dilution; MA5-25690; Thermo Fisher Scientific, USA), anti-CDC16 (1:1000 dilution; MA5-32406; Thermo Fisher Scientific, USA), anti-PTPRA (dilution; 1:1000; PA5-15522; Thermo Fisher Scientific, USA), anti-MZT2B (Santa Cruz Biotechnology, USA; dilution 1:1000; sc-514516), anti-UBE2T (1:500; MA5-25226; Thermo Fisher Scientific, USA) and ACTB (dilution 1:250; Ac-15), ACTB was used as a loading reference control.

Immunohistochemistry

Immunoreactivity of proteins encoded by the selected genes TTI12 (1:50 dilution; MA5-25018; Thermo Fisher Scientific, USA), CDKN3 (dilution 1:150; MA5-25690; Thermo Fisher Scientific, USA), CDC16 (dilution 1:150; MA5-32406; Thermo Fisher Scientific, USA), PTPRA (dilution; 1: 100; PA5-15522; Thermo Fisher Scientific, USA), MZT2B (Santa Cruz Biotechnology, USA; 1:1000 dilution; sc-514516), UBE2T (1:50; MA5-25226; Thermo Fisher Scientific, USA) was evaluated by immunohistochemistry on paraffin-embedded tissue sections, as suggested by Calcagno et al. [11].

Statistical analysis

The validation data are shown as the frequency, median, and interquartile range (IQR). The Mann-Whitney test was used to investigate the possible associations between gene mRNA or protein expression and categorical variables, such as immunoreactivity and clinicopathological features. An association between categorical variables was analyzed using the χ2 test, a p-value ≤ 0.05 was considered significant. The median and interquartile range (IQR) were used to assess the degree of data dispersion around the centrality measure. The difference between the upper and lower quartiles was determined for the interquartile range. Heat maps were used to show hierarchical groupings of the gene expression profile of the samples, using the Z-score metric. The Kaplan-Meier estimator and the log-rank test were used to estimate the survival probability of the high and low expression groups defined by the cut-off points obtained by the Area-Under-the-Curve (AUC) for the TTI12, CDC16, UBE2T and MZT2B. A 95% confidence level was considered and p <0.001. All statistics associated with clinical samples were performed using R (https://www.r-project.org).

Data availability

The gene expression data used to support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) repository under the access number GSE81265 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE 81265).

AUTHOR CONTRIBUTIONS

Conceptualization, J.H.d.S.M., H.F.R. and C.d.F.A.M.-N.; Methodology, J.H.d.S.M., C.d.F.A.M.-N., H.F.R., R.d.M.M., R.M.d.S., R.P.d.T., B.D.K.M.C. and P.C.S.; Software, J.H.d.S.M.; Formal analysis, J.H.d.S.M., C.d.F.A.M.-N. and R.M.R.B.; Investigation, J.H.d.S.M., C.d.F.A.M.-N. and R.M.R.B.; Data curation, J.H.d.S.M. and R.M.R.B.; Validation, R.d.M.M., R.M.d.S., R.P.d.T., B.D.K.M.C., P.C.S., P.P.A. and R.M.R.B;
Visualization, J.H.d.S.M., H.F.R., C.d.F.A.M.-N. and R.M.R.B.; Writing—original draft, J.H.d.S.M. and H.F.R; Writing—review & editing, J.H.d.S.M. and C.d.F.A.M.-N.; Resources, J.H.d.S.M., H.F.R., C.d.F.A.M.-N. and R.M.R.B.; Project administration, P.C.S., P.P.A. and R.M.R.B.; Funding acquisition, R.M.R.B.; Supervision, R.M.R.B.

ACKNOWLEDGMENTS

We are grateful to all patients, researchers and staff at the Ophir Loyola Hospital and Universidade Federal do Pará.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

The authors thank the financial support of this study, supported by Coordination for the Improvement of Higher Education Personnel (CAPES; to J.H.d.S.M.) and Brazilian funding agencies National Counsel of Technological and Scientific Development (CNPq; grants #305220/2013-6 to H.F.R. and to R.M.R.B.), as well as PROPESP/UFPA for payment of the publication fees.

REFERENCES

1. Sitarz R, Skierucha M, Mielko J, Offerhaus GJ, Maciejewski R, Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res. 2018; 10:239–48. https://doi.org/10.2147/CMAR.S149619 PMID:29445300

2. Ferro A, Peleteiro B, Malvezzi M, Bosetti C, Bertuccio P, Levi F, Negri E, La Vecchia C, Lunet N. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. Eur J Cancer. 2014; 50:1330–44. https://doi.org/10.1016/j.ejca.2014.01.029 PMID:24650579

3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68:394–424. https://doi.org/10.3322/caac.21492 PMID:30207593

4. Carcas LP. Gastric cancer review. J Carcinog. 2014; 13:14. https://doi.org/10.4103/1477-3163.146506 PMID:25589897

5. McLean MH, El-Omar EM. Genetics of gastric cancer. Nat Rev Gastroenterol Hepatol. 2014; 11:664–74. https://doi.org/10.1038/nrgastro.2014.143 PMID:25134511

6. Orditura M, Galizia G, Sforza V, Gambardella V, Fabozzi A, Laterza MM, Andreozzi F, Ventriglia J, Savastano B, Mabilia A, Lieto E, Ciardiello F, De Vita F. Treatment of gastric cancer. World J Gastroenterol. 2014; 20:1635–49. https://doi.org/10.3748/wjg.v20.i7.1635 PMID:24587643

7. Calcagno DQ, Freitas VM, Leal MF, de Souza CR, Demachki S, Montenegro R, Assumpção PP, Khayat AS, Smith Mde A, dos Santos AK, Burbano RR. MYC, FBXW7 and TP53 copy number variation and expression in gastric cancer. BMC Gastroenterol. 2013; 13:141. https://doi.org/10.1186/1471-230X-13-141 PMID:24053468

8. Calcagno DQ, Leal MF, Taken SS, Assumpção PP, Demachki S, Smith Mde A, Burbano RR. Aneuploidy of chromosome 8 and C-MYC amplification in individuals from northern Brazil with gastric adenocarcinoma. Anticancer Res. 2005; 25:4069–74. PMID:16309200

9. Costa Guimarães A, Gonçalves Quintana L, Ferreira Leal M, Satomi Takeno S, Pimentel Assumpção P, Moura Lima E, Salim Khayat A, Suchi Chen E, de Arruda Cardoso Smith M, Rodrigues Burbano R. Aneuploidy of chromosome 8 detected by fluorescence in situ hybridisation in ACP01 cell line gastric adenocarcinoma. Clin Exp Med. 2006; 6:129–38. https://doi.org/10.1007/s10238-006-0108-5 PMID:17061062

10. Lima EM, Rissino JD, Harada ML, Assumpção PP, Demachki S, Guimarães AC, Casartelli C, Smith MA, Burbano RR. Conventional cytogenetic characterization of a new cell line, ACP01, established from a primary human gastric tumor. Braz J Med Biol Res. 2004; 37:1831–38. https://doi.org/10.1590/s0100-879x2004001200008 PMID:15558189

11. Calcagno DQ, Leal MF, Seabra AD, Khayat AS, Chen ES, Demachki S, Assumpção PP, Faria MH, Rabenhorst SH, Ferreira MV, de Arruda Cardoso Smith M, Burbano RR. Interrelationship between chromosome 8 aneuploidy, C-MYC amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma. World J Gastroenterol. 2006; 12:6207–11. https://doi.org/10.3748/wjg.v12.i38.6207 PMID:17036397

12. Ribeiro HF, Alcântara DF, Matos LA, Sousa JM, Leal MF, Smith MA, Burbano RR, Bahia MO. Cytogenetic
characterization and evaluation of c-MYC gene amplification in PG100, a new Brazilian gastric cancer cell line. Braz J Med Biol Res. 2010; 43:717–21.

https://doi.org/10.1590/s0100-879x20100007500068
PMID:20658094

13. Panani AD. Cytogenetic and molecular aspects of gastric cancer: clinical implications. Cancer Lett. 2008; 266:99–115.

https://doi.org/10.1016/j.canlet.2008.02.053
PMID:18381231

14. Liu X, Cai H, Huang H, Long Z, Shi Y, Wang Y. The prognostic significance of apoptosis-related biological markers in Chinese gastric cancer patients. PLoS One. 2011; 6:e29670.

https://doi.org/10.1371/journal.pone.0029670
PMID:22163420

15. Choi JS, Seo J, Jung EJ, Kim EJ, Lee GK, Kim WH. c-MYC amplification in mucinous gastric carcinoma: a possible genetic alteration leading to deeply invasive tumors. Anticancer Res. 2012; 32:5031–37.

PMID:23155275

16. Leal MF, Ribeiro HF, Rey JA, Pinto GR, Smith MC, Moreira-Nunes CA, Assumpção PP, Lamarão LM, Calcagno DQ, Montenegro RC, Burbano RR. YWHAE silencing induces cell proliferation, invasion and migration through the up-regulation of CDC25B and MYC in gastric cancer cells: new insights about YWHAE role in the tumor development and metastasis process. Oncotarget. 2016; 7:85393–410.

https://doi.org/10.18632/oncotarget.13381
PMID:27863420

17. Mello AA, Leal MF, Rey JA, Pinto GR, Lamarão LM, Montenegro RC, Alves AP, Assumpção PP, Borges Bd N, Smith MC, Burbano RR. Deregulated expression of SRC, LYN and CKB kinases by DNA methylation and its potential role in gastric cancer invasiveness and metastasis. PLoS One. 2015; 10:e0140492.

https://doi.org/10.1371/journal.pone.0140492
PMID:26460485

18. Tansey WP. Mammalian MYC Proteins and Cancer. New J Sci. 2014; 2014:1–27.

https://doi.org/10.1155/2014/757534

19. Zhang L, Hou Y, Ashktorab H, Gao L, Xu Y, Wu K, Zhai J, Zhang L. The impact of C-MYC gene expression on gastric cancer cell. Mol Cell Biochem. 2010; 344:125–35.

https://doi.org/10.1007/s11010-010-0536-0
PMID:20737197

20. Unlocking the mysterious mechanisms of myc. Nat Med. 2013; 19:26–27.

https://doi.org/10.1038/nm.3060
PMID:23296005

21. Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev. 2014; 23:700–13.

https://doi.org/10.1158/1055-9965.EPI-13-1057
PMID:24618998

22. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014; 513:202–9.

https://doi.org/10.1038/nature13480
PMID:25079317

23. Figueiredo C, Camargo MC, Leite M, Fuentes-Pananá EM, Rabkin CS, Machado JC. Pathogenesis of Gastric Cancer: Genetics and Molecular Classification. Curr Top Microbiol Immunol. 2017; 400:1–304.

https://doi.org/10.1007/978-3-319-50520-6_12
PMID:28124158

24. Cools-Lartigue J, Baker L, Ferri LE. Molecular Mechanisms in Gastric Carcinogenesis. In: Strong VE, editor. Gastric Cancer. Springer, Cham. 2015. pp. 35–56.

https://doi.org/10.1007/978-3-319-15826-6_3

25. Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, Chan KH, Chan AS, Tsui WY, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014; 46:573–82.

https://doi.org/10.1038/ng.2983
PMID:24816253

26. Leal MF, Martins do Nascimento JL, da Silva CE, Vita Lamarão MF, Calcagno DQ, Khayat AS, Assumpção PP, Cabral IR, de Arruda Cardoso Smith M, Burbano RR. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. Cancer Genet Cytogenet. 2009; 195:85–91.

https://doi.org/10.1016/j.cancergencyto.2009.04.020
PMID:19837275

27. Wisnieski F, Calcagno DQ, Leal MF, dos Santos LC, Gigek Cde O, Chen ES, Pontes TB, Assumpção PP, de Assumpção MB, Demachki S, Burbano RR, Smith Mde A. Reference genes for quantitative RT-PCR data in gastric tissues and cell lines. World J Gastroenterol. 2013; 19:7121–28.

https://doi.org/10.3748/wjg.v19.i41.7121
PMID:24222956

28. Leal MF, Calcagno DQ, Borges da Costa Jde F, Silva TC, Khayat AS, Chen ES, Assumpção PP, de Arruda Cardoso Smith M, Burbano RR. MYC, TP53, and chromosome 17 copy-number alterations in multiple gastric cancer cell lines and in their parental primary tumors. J Biomed Biotechnol. 2011; 2011:631268.

https://doi.org/10.1155/2011/631268
PMID:21528007
31. de Souza CR, Leal MF, Calcagno DQ, Costa Sozinho EK, Borges Bdo N, Montenegro RC, Dos Santos AK, Dos Santos SE, Ribeiro HF, Assumpção PP, de Arruda Cardoso Smith M, Burbano RR. MYC deregulation in gastric cancer and its clinicopathological implications. PLoS One. 2013; 8:e64420. https://doi.org/10.1371/journal.pone.0064420 PMID:23717612

32. da Costa Jde F, Leal MF, Silva TC, Andrade Junior EF, Rezende AP, Muniz JA, Lacreta Junior AC, Assumpção PP, Calcagno DQ, Demachki S, Rabenhorst SH, Smith Mde A, Burbano RR. Experimental gastric carcinogenesis in cebus apella nonhuman primates. PLoS One. 2011; 6:e21988. https://doi.org/10.1371/journal.pone.0021988 PMID:21811552

33. Silva TC, Leal MF, Calcagno DQ, de Souza CR, Khayat AS, dos Santos NP, Montenegro RC, Rabenhorst SH, Nascimento MQ, Assumpção PP, de Arruda Cardoso Smith M, Burbano RR. hTERT, MYC and TP53 deregulation in gastric preneoplastic lesions. BMC Gastroenterol. 2012; 12:85. https://doi.org/10.1186/1471-230X-12-85 PMID:22768805

34. Burbano RR, Assumpção PP, Leal MF, Calcagno DQ, Guimarães AC, Khayat AS, Takeno SS, Chen ES, De Arruda Cardoso Smith M. C-MYC locus amplification as metastasis predictor in intestinal-type gastric adenocarcinomas: CGH study in Brazil. Anticancer Res. 2006; 26:2909–14. PMID:16886612

35. Calcagno DQ, Leal MF, Demachki S, Araújo MT, Freitas FW, Oliveira e Souza D, Assumpção PP, Ishak G, de Arruda Cardoso Smith M, Burbano RR. MYC in gastric carcinoma and intestinal metaplasia of young adults. Cancer Genet Cytogenet. 2010; 202:63–66. https://doi.org/10.1016/j.cancergenetcyto.2010.05.020 PMID:20804924

36. Costa Raiol LC, Figueira Silva EC, Mendes da Fonseca D, Leal MF, Guimarães AC, Calcagno DQ, Khayat AS, Assumpção PP, de Arruda Cardoso Smith M, Burbano RR. Interrelationship between MYC gene numerical aberrations and protein expression in individuals from northern Brazil with early gastric adenocarcinoma. Cancer Genet Cytogenet. 2008; 181:31–35. https://doi.org/10.1016/j.cancergenetcyto.2007.10.011 PMID:18262050

37. Zhang L, Du X, Wei S, Li D, Li Q. A comprehensive transcriptomic view on the role of SMAD4 gene by RNAi-mediated knockdown in porcine follicular granulosa cells. Reproduction. 2016; 152:81–89. https://doi.org/10.1530/REP-16-0034 PMID:27107035

38. Deisenroth C, Black MB, Pendse S, Pluta L, Witherspoon SM, McMullen PD, Thomas RS. MYC is an early response regulator of human adipogenesis in adipose stem cells. PLoS One. 2014; 9:e114133. https://doi.org/10.1371/journal.pone.0114133 PMID:25437437

39. Xu W, Yang Z, Lu N. Molecular targeted therapy for the treatment of gastric cancer. J Exp Clin Cancer Res. 2016; 35:1. https://doi.org/10.1186/s13046-015-0276-9 PMID:26728266

40. Riquelme I, Saavedra K, Espinoza JA, Weber H, Garcia P, Nervi B, Garrido M, Corvalán AH, Roa JC, Bizama C. Molecular classification of gastric cancer: towards a pathway-driven targeted therapy. Oncotarget. 2015; 6:24750–79. https://doi.org/10.18632/oncotarget.4990 PMID:26267324

41. Moreira-Nunes CA, Barros MB, do Nascimento Borges B, Montenegro RC, Lamarão LM, Ribeiro HF, Bona AB, Assumpção PP, Rey JA, Pinto GR, Burbano RR. Genetic screening analysis of patients with hereditary diffuse gastric cancer from northern and northeastern Brazil. Hered Cancer Clin Pract. 2014; 12:18. https://doi.org/10.1186/1897-4287-12-18 PMID:25180051

42. Alexandrova N, Niklinski J, Bliskovsky V, Ottersen GA, Blake M, Kaye FJ, Zajac-Kaye M. The n-terminal domain of c-myc associates with alpha-tubulin and microtubules in vivo and in vitro. Mol Cell Biol. 1995; 15:5188–95. https://doi.org/10.1128/mcb.15.9.5188 PMID:7651436

43. Niklinski J, Claassen G, Meyers C, Gregory MA, Allegra CJ, Kaye FJ, Hann SR, Zajac-Kaye M. Disruption of Myc-tubulin interaction by hyperphosphorylation of c-Myc during mitosis or by constitutive hyperphosphorylation of mutant c-Myc in Burkitt’s lymphoma. Mol Cell Biol. 2000; 20:5276–84.
44. Wasylyk C, Zambrano A, Zhao C, Brants J, Abecassis J, Schalken JA, Rogatsch H, Schaefer G, Pycha A, Klocker H, Wasylyk B. Tubulin tyrosine ligase like 12 links to prostate cancer through tubulin posttranslational modification and chromosome ploidy. Int J Cancer. 2010; 127:2542–53. https://doi.org/10.1002/ijc.25261 PMID:20162578

45. Wen R, Xiao Y, Zhang Y, Yang M, Lin Y, Tang J. Identification of a novel transcript isoform of the TTLL12 gene in human cancers. Oncol Rep. 2016; 36:3172–80. https://doi.org/10.3892/or.2016.5135 PMID:27748896

46. Yang S, Liang Y, Qian H, Li Q. TTLL12 expression in ovarian cancer correlates with a poor outcome. Int J Clin Exp Pathol. 2020; 13:239–47. PMID:32211104

47. den Hoed CM, Kuipers EJ. Gastric cancer: how can we reduce the incidence of this disease? Curr Gastroenterol Rep. 2016; 18:34. https://doi.org/10.1007/s11894-016-0506-0 PMID:27184043

48. Tilli TM, Castro Cda S, Tusznyski JA, Carels N. A strategy to identify housekeeping genes suitable for analysis in breast cancer diseases. BMC Genomics. 2016; 17:639. https://doi.org/10.1186/s12864-016-2946-1 PMID:27526934

49. Zang S, Guo R, Xing R, Zhang L, Li W, Zhao M, Fang J, Hu F, Kang B, Ren Y, Zhuang Y, Liu S, Wang R, et al. Identification of differentially-expressed genes in intestinal gastric cancer by microarray analysis. Genomics Proteomics Bioinformatics. 2014; 12:276–83. https://doi.org/10.1016/j.gpb.2014.09.004 PMID:25500430

50. Berth F, Bollschweiler E, Drebber U, Hoelscher AH, Moenig S. Pathohistological classification systems in gastric cancer: diagnostic relevance and prognostic value. World J Gastroenterol. 2014; 20:5679–84. https://doi.org/10.3748/wjg.v20.i19.5679 PMID:24914328

51. Heichman KA, Roberts JM. CDC16 controls initiation at chromosome replication origins. Mol Cell. 1998; 1:457–63. https://doi.org/10.1016/s1097-2765(00)80046-5 PMID:960930

52. Liu L, Guo K, Liang Z, Li F, Wang H. Identification of candidate genes that may contribute to the metastasis of prostate cancer by bioinformatics analysis. Oncol Lett. 2018; 15:1220–28. https://doi.org/10.3892/ol.2017.7404 PMID:29399176

53. Kim EJ, Park MK, Byun HJ, Kang GJ, Yu L, Kim HJ, Shim JG, Lee H, Lee CH. YdjC chitooligosaccharide deacetylase homolog induces keratin reorganization in lung cancer cells: involvement of interaction between YDJC and CDC16. Oncotarget. 2018; 9:22915–28. https://doi.org/10.18632/oncotarget.25145 PMID:29796162

54. Wang Q, Moyret-Lalle C, Couzon F, Surbiguet-Cliffe C, Saurin JC, Lorca T, Navarro C, Puisieux A. Alterations of anaphase-promoting complex genes in human colon cancer cells. Oncogene. 2003; 22:1486–90. https://doi.org/10.1038/sj.onc.1206224 PMID:12629511

55. Yu H, Xiang P, Pan Q, Huang Y, Xie N, Zhu W. Ubiquitin-conjugating enzyme E2T is an independent prognostic factor and promotes gastric cancer progression. Tumour Biol. 2016; 37:11723–32. https://doi.org/10.1007/s13277-016-5020-3 PMID:27020591

56. Luo C, Yao Y, Yu Z, Zhou H, Guo L, Zhang J, Cao H, Zhang G, Li Y, Jiao Z.UBE2T knockdown inhibits gastric cancer progression. Oncotarget. 2017; 8:32639–54. https://doi.org/10.18632/oncotarget.15947 PMID:28427240

57. Li Y, Ji S, Fu LY, Jiang T, Wu D, Meng FD. Knockdown of cyclin-dependent kinase inhibitor 3 inhibits proliferation and invasion in human gastric cancer cells. Oncol Res. 2017; 25:721–31. https://doi.org/10.3727/096504016X14772375848616 PMID:27983933

58. Fan C, Chen L, Huang Q, Shen T, Welsh EA, Teer JK, Cai J, Cress WD, Wu J. Overexpression of major CDK3 transcripts is associated with poor survival in lung adenocarcinoma. Br J Cancer. 2015; 113:1735–43. https://doi.org/10.1038/bjc.2015.378 PMID:26554648

59. Wu CW, Kao HL, Li AF, Chi CW, Lin WC. Protein tyrosine-phosphatase expression profiling in gastric cancer tissues. Cancer Lett. 2006; 242:95–103. https://doi.org/10.1016/j.canlet.2005.10.046 PMID:16338072

60. Faria AVS, Andrade SS, Reijm AN, Spaander M, de Maat PMP, Peppelenbosch MP, Ferreira-Halder CV, Fuhler GM. Targeting Tyrosine Phosphatases by 3-Bromopyruvate Overcomes Hyperactivation of Platelets from Gastrointestinal Cancer Patients. J Clin Med. 2020; 9:2625. https://doi.org/10.3390/jcm9082625 PMID:32823601

61. Du Y, Grandis JR. Receptor-type protein tyrosine phosphatases in cancer. Chin J Cancer. 2015; 34:61–9. https://doi.org/10.5732/jjc.014.10146 PMID:25322863
62. Liu X, Wu J, Zhang D, Bing Z, Tian J, Ni M, Zhang X, Meng Z, Liu S. Identification of potential key genes associated with the pathogenesis and prognosis of gastric cancer based on integrated bioinformatics analysis. Front Genet. 2018; 9:265. https://doi.org/10.3389/fgene.2018.00265 PMID:30065754

63. Bao B, Zheng C, Yang B, Jin Y, Hou K, Li Z, Zheng X, Yu S, Zhang X, Fan Y, Qu X, Liu Y, Che X. Identification of subtype-specific three-gene signature for prognostic prediction in diffuse type gastric cancer. Front Oncol. 2019; 9:1243. https://doi.org/10.3389/fonc.2019.01243 PMID:31803620

64. Zhang W, Wan YW, Allen GI, Pang K, Anderson ML, Liu Z. Molecular pathway identification using biological network-regularized logistic models. BMC Genomics. 2013 (Suppl 8); 14:S7. https://doi.org/10.1186/1471-2164-14-S8-S7 PMID:24564637

65. Audic S, Claverie JM. The significance of digital gene expression profiles. Genome Res. 1997; 7:986–95. https://doi.org/10.1101/gr.7.10.986 PMID:9331369

66. Reimand J, Arak T, Adler P, Kolberg L, Reisberg S, Peterson H, Vilo J. g:Profiler-a web server for functional interpretation of gene lists (2016 update). Nucleic Acids Res. 2016; 44:W83–89. https://doi.org/10.1093/nar/gkw199 PMID:27098042

67. Korotkevich G, Sukhov V, Sergushichev A. Fast gene set enrichment analysis. bioRxiv. 2016. https://doi.org/10.1101/060012

68. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012; 16:284–87. https://doi.org/10.1089/omi.2011.0118 PMID:22455463

69. Washington K. 7th edition of the AJCC cancer staging manual: stomach. Ann Surg Oncol. 2010; 17:3077–79. https://doi.org/10.1245/s10434-010-1362-z PMID:20882416

70. de Souza CR, de Oliveira KS, Ferraz JJ, Leal MF, Calcagno DQ, Seabra AD, Khayat AS, Montenegro RC, Alves AP, Assumpção PP, Smith MC, Burbano RR. Occurrence of helicobacter pylori and epstein-barr virus infection in endoscopic and gastric cancer patients from northern Brazil. BMC Gastroenterol. 2014; 14:179. https://doi.org/10.1186/1471-230X-14-179 PMID:25318991

71. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001; 25:402–08. https://doi.org/10.1006/meth.2001.1262 PMID:11846609
SUPPLEMENTARY MATERIALS

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1–6.

Supplementary Table 1. A total of 5,327 DEGs identified in the ACP 02 lineage, of which 4,098 DEGs with a downregulated profile and 1,229 with an upregulated profile. MYC-siRNA: (2M) vs. Control-siRNA: (2C).

Supplementary Table 2. A total of 4,117 DEGs identified in the ACP03 lineage, of which 843 DEGs with a downregulated profile and 3,274 with an upregulated profile. MYC-siRNA: (3M) vs. Control-siRNA: (3C).

Supplementary Table 3. Clinical-pathological variables and gene expression of TTLL12, CDKN3 and CDC16 in gastric cancer.

Supplementary Table 4. Clinicopathological variables and gene expression of PTPRA, MZT2B and UBE2T in gastric cancer.

Supplementary Table 5. Results of MYC silencing on gene and protein expression in ACP02 and ACP03.

Supplementary Table 6. GSEA analysis of the DEGs in ACP02 and ACP03.