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ROLE OF REPRIMO-LIKE AS A TUMOR SUPPRESSOR AND CANDIDATE BIOMARKER IN GASTRIC CANCER

Dissertation presented for the degree of
Doctor of Philosophy (PhD) in Medical Sciences

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I. ABSTRACT

Gastric cancer is one of the leading causes of cancer death. Elucidating the molecular bases of gastric cancer might contribute to develop opportune diagnostic and therapeutic strategies. *Reprimo-like (RPRML)* is a poorly characterized member of the *Reprimo* gene family. The founding member of this family, *Reprimo (RPRM*), is reported to be a putative tumor suppresser gene, downregulated by DNA methylation in gastric cancer, and a candidate biomarker for gastric cancer diagnosis. RPRML shares high homology with RPRM and is vastly conserved among vertebrates. We hypothesized that *RPRML acts as a tumor suppressor gene, downregulated by DNA methylation in gastric cancer, and circulates as methylated DNA in gastric cancer patients.*

RPRML transcript expression was analyzed in 32 matched-pairs of stomach adenocarcinomas and non-tumor adjacent mucosa (NTAM) from the TCGA STAD dataset. Protein expression was assessed immunohistochemically in 17 matched-pairs of stomach adenocarcinoma and NTAM tissues. Its clinical significance in gastric cancer was evaluated by IHC staining in a cohort of 90 gastric cancer patients and association analysis with clinicopathologic data, overall survival (OS), and status of molecular markers. The role of *RPRML* in tumor suppression was investigated *in vitro* through stable overexpression and cell sorting of RPRML coupled to GFP or GFP alone in the AGS cell line. Functional assays included MTS assay, Ki67 and cleaved-caspase3 immunofluorescence, flow cytometric analysis of cell cycle, colony formation and soft agar assays. Methylation-associated silencing was evaluated *in vitro* by 5-azacytidine assay and direct bisulfite sequencing. Detection of methylated *RPRML* DNA in plasma samples was assessed by MSP assay in 21 gastric cancer patients and 36 altruistic blood donors; and by MethylLight assay in 25 gastric cancer and 64 low risk OLGA patients.

RPRML transcript and protein expression were significantly downregulated in gastric cancer tissues compared to matched NTAM (p=0.01681 and p=0.001, respectively). RPRML protein expression was a significant prognostic factor for overall survival in advanced-stage gastric cancer patients (HR 0.07, 95%CI: 0.01-0.46, p=0.005). Overexpression of *RPRML* in the AGS cell line significantly inhibited cell proliferation (p<0.05), reduced clonogenic capacity (p=0.007), decreased anchorage-independent growth (p=0.001), and arrested progression of cell cycle through G2/M phase (p<0.05), but did not affect apoptosis. Inhibition of DNA methylation restored *RPRML* transcript expression in three of four gastric cancer cell lines, with prior undetectable expression. MSP assay detected a higher rate of methylated

RPRML DNA in gastric cancer patients than in blood donors (p=0.002). MethyLight assay significantly discriminated gastric cancer patients from low risk OLGA patients (AUC: 0.729, 95%CI: 0.599-0.860, p=0.001). With a cutoff value of 19.0 copies/ml of plasma, sensitivity was 56.0% (95%CI:34.93-75.60) and specificity of 87.5% (95%CI: 76.85-94.45).

Our findings indicate that *RPRML* is downregulated in gastric cancer, and low expression is a risk factor for poor prognosis in advanced stages of disease. Moreover, ectopic overexpression of *RPRML in vitro* inhibited biological characteristics associated with tumor progression, suggesting a tumor suppressor role in gastric cancer. Restoration of RPRML transcript expression by 5-Azacytidine suggests that DNA methylation plays an important role in the regulation of RPRML expression. Detection of circulating methylated RPRML DNA in plasma is proposed as a potential biomarker for non-invasive detection of gastric cancer.

II. INTRODUCTION

Gastric cancer remains the fifth most frequently diagnosed cancer globally and the third leading cause of cancer-related death (Ferlay et al., 2014, 2018). Although incidence has been declining in developed countries during the last decades, the absolute number of cases will continue to increase due to the aging and growth of the population (Arnold et al., 2020). Thus, it remains a major public health challenge worldwide.

1. Pathogenesis and prognosis of gastric cancer

A small percentage of gastric cancers (1-3%) has been associated to hereditary causes, however, the majority of cases develops sporadically as a consequence of multiple interacting factors. Approximately 95% of gastric cancers correspond to stomach adenocarcinomas (Piazuelo & Correa, 2013). The most relevant etiological agent is infection with *Helicobacter pylori (H. pylori),* specially with the oncogenic strains cag-A and vac-A; and secondly, infection with Epstein Barr Virus (Piazuelo & Correa, 2013). Interaction of this pathogens with host and environmental predisposing factors, can trigger a multistep, long –lasting and mostly asymptomatic process of gastric mucosal transformation.

The carcinogenic process usually develops through the progression of sequential precancerous lesions, known as The Correa's cascade (Correa & Piazuelo, 2012), that progress from non-atrophic chronic gastritis, to multifocal atrophic gastritis (MAG), intestinal metaplasia (IM), and finally dysplasia. Each step with an increased individual risk for gastric cancer (de Vries et al., 2008). Accordingly, the degree and extension of atrophic changes significantly correlates with gastric cancer risk. The Operative Link for Gastritis Assessment (OLGA) has stratified five histological stages according to their cancer risk: OLGA 0, I, II, III and IV (Rugge et al., 2007). OLGA 0, I and II are considered low risk lesions, while OLGA III and IV possess a higher risk for cancer progression (HR = 712.4 and HR = 1450.7, respectively). Follow-up is generally recommended for this patients (Rugge et al., 2018).

When gastric cancer is established, the patients' prognosis depends mainly on the clinical stage at diagnosis (Nashimoto et al., 2013). The American Joint Committee on Cancer (AJCC) has developed a precise stage classification system, incorporating the tumor depth (T), lymph node involvement (N) and the presence of distant metastases (M) to assess patient's prognosis and appropriate treatment (Amin et al., 2017). Early stage gastric cancer (TNM Stage I-II) has 5-year survival rates between 60-95%, while

advanced stage gastric cancer (TNM Stages III-IV) has less than 10% of survival rates (Y. Chen et al., 2019; H. Wang et al., 2018). Still, more than 80% of all cases are diagnosed in advanced stages (Price et al., 2012) reflecting a broad deficiency in early diagnosis approaches. Thus, elucidating the molecular mechanisms driving the progression of precancerous and cancerous lesions has become crucial for the development of novel biomarkers and precision medicine strategies.

2. Molecular characterization of gastric cancer

In recent years, large-scale clinical and molecular profiling projects have contributed to better understand the molecular basis of gastric cancer. In this scenario, the comprehensive molecular characterization of tumors by The Cancer Genome Atlas (TCGA) Research Network (Cancer Genome Atlas Research Network, 2014) has profiled nearly 400 gastric tumor samples from distinct ethnic backgrounds at the genomic, transcriptomic, methylome and proteomic level, distinguishing four molecular subtypes of gastric cancer: EBV-subtype, is characterized by enriched in PIK3CA mutations, PD-L1 and PD-L2 overexpression, and extreme levels of DNA methylation, also known as "CpG island methylator phenotype" (CIMP); Microsatellite-unstable (MSI)-subtype has elevated rates of mutations, typically alterations on MHC class I genes, and CIMP; Genomically Stable-subtype has common CDH1 and RHO-family alterations; and Chromosomally-Unstable -subtype usually harbors TP53 mutations, aneuploidy, and activation of RTK-RAS pathway.

Remarkably, genetic alterations in cancer-related genes (i.e. tumor suppressor- and oncogenes) were not as frequent as expected and displayed high heterogeneity. Rather, epigenetic alterations were recurrent and common to all subtypes, suggesting a major role in driving gastric carcinogenesis and a promising target for biomarkers studies (Cancer Genome Atlas Research Network, 2014; Padmanabhan et al., 2017).

3. DNA methylation in gastric cancer

DNA methylation is an epigenetic mechanism that controls gene expression in a time- and tissue-specific manner during normal cellular processes. It consists in the covalent addition of a methyl group to CpG dinucleotides (CpG sites) that are generally clustered near gene promoters (CpG islands), affecting local chromatin structure and transcription factor binding (Bernstein et al., 2007). Aberrant patterns of

DNA methylation have been extensively reported in different cancer types, altering expression of several cancer-related genes (i.e. tumor suppressor and oncogenes), and directly contributing to cancer development (Padmanabhan et al., 2017).

A growing body of evidence has suggested that DNA methylation plays a major role in gastric carcinogenesis. In fact, both H.*pylori* and EBV pathogens have shown to induce promoter methylation and silencing of several tumor suppressor genes in gastric mucosa (Maeda et al., 2017; Matsusaka et al., 2014). Moreover, gene-specific methylation found in precancerous lesions has been associated to an increased gastric cancer risk (Sandoval-Bórquez et al., 2015).

Numerous tumor suppressor genes are known to be downregulated by DNA methylation in gastric cancer (Padmanabhan et al., 2017). In addition, several studies have demonstrated that methylated DNA can be detected in different biological fluids and is thought to be derived from cancer tissues (D. Chen et al., 2020). Therefore, DNA methylation is considered a promising biomarker for non-invasive detection of gastric cancer.

4. The *Reprimo* gene family

The *Reprimo* gene family emerged from a common ancestor after two rounds of whole genome duplication (WGD) events during early vertebrates evolution (Wichmann et al., 2016). Members of this family have been differentially retained among distinct vertebrate species. Nonetheless, only two members have been retained in humans: *Reprimo* (*RPRM*) and *Reprimo-like* (*RPRML*).

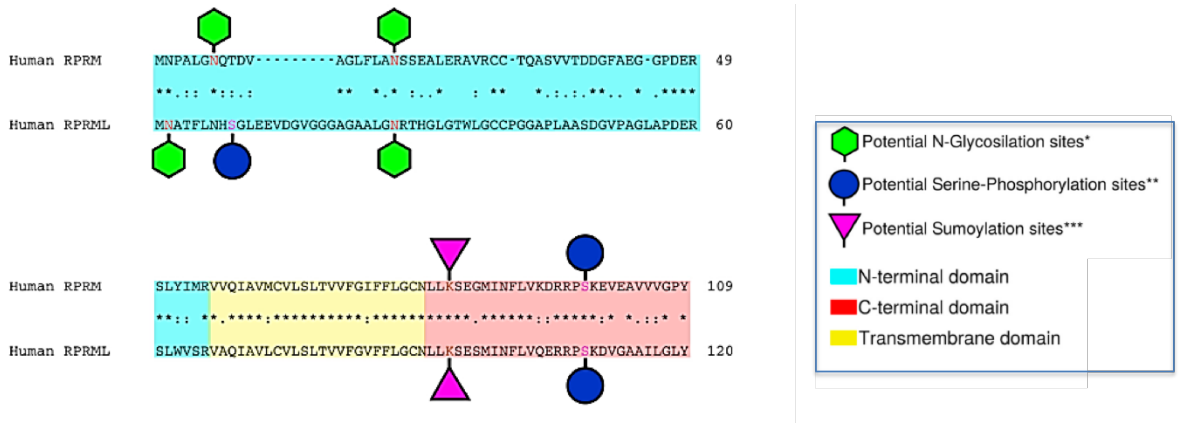
RPRM and *RPRML* are both intronless genes located in the reverse strand of chromosome 2 and 17, respectively. *RPRM*, the founding member of this family, was first identified by Ohki *et al*. in the year 2000 (Ohki et al., 2000) as a p53-induced cell cycle arrest mediator, that inhibits nuclear translocation of Cdc2-CyclinB1 complex during G2 phase. Although the exact molecular interactions involved in *RPRM*-signaling remain unknown, extensive evidence has suggested that *RPRM* acts as tumor suppressor gene. For instance, *RPRM* exogenous overexpression results in reduced colony formation and anchorage-independent growth, as well as increased apoptosis in gastric cancer cell lines (Ooki et al., 2013; Saavedra et al., 2015). Similar effects have been reported In pituitary and breast cancer cell lines (Xu et al., 2012), (Buchegger et al., 2016). Correspondingly, *RPRM* overexpression in mouse xenografts models results in a

reduction of tumor weight and volume (Ooki et al., 2013). Moreover, in gastric cancer patients, *RPRM* silencing has been associated with increased depth of tumor invasion, lymphatic vessel invasion, lymph node metastasis, and progression from TNM stage I to TNM stages II-IV (Luo et al., 2011; Saavedra et al., 2015).

RPRM has been reported to be methylated and silenced in a broad range of cancers and cancer cell lines, including breast (Buchegger et al., 2016), pituitary (Xu et al., 2012) and renal (Morris et al., 2010) cancers. Particularly, research by our group has found that *RPRM* is consistently methylated and silenced in a tumor-specific manner in gastric cancer. Furthermore, inhibition of DNA methylation *in vitro* restores *RPRM* transcript expression (Bernal et al., 2008), suggesting a causal link for DNA methylation in *RPRM* transcriptional silencing. In addition, methylated DNA of *RPRM* was found in circulation of gastric cancer patients, significantly discriminating gastric cancer from blood donors (Bernal et al., 2008). Therefore, DNA methylation of *RPRM* has been proposed as a candidate biomarker for gastric cancer detection (Saliminejad et al., 2020; Wen et al., 2017)

RPRML, the other member of this family, has been less studied so far. In 2019, Stanic *et al.*(Stanic et al., 2019) reported for the first time a physiological role of *RPRML*. By knocking down RPRML on a zebrafish model, they showed that RPRML was required for the formation of hematopoietic progenitors during the embryonic development of zebrafish. In addition, they reported increased caspase-dependent apoptosis in RPRML knockdown embryos. A functional role in humans is yet to be determined.

RPRM and *RPRML* share 52.5% aminoacid sequence identity in humans, suggesting they might have similar biological functions (Wichmann et al., 2016). They are also highly conserved among species preserving a predicted serine-phosphorylation site and a SUMOylation site (Amigo et al., 2018), known to be crucial in several cellular processes including DNA repair, cell cycle progression, and cell differentiation (Komiya et al., 2017), thus, suggesting a key functional role. Also, both genes have transmembrane domain motifs, suggesting they might be integral components of the cell membrane (UniprotKB, n.d.). While the N-terminal portion differ widely in sequence, both genes maintain consensus sequences for two N-glycosylation sites, a post-translational modification well-known for its role in protein folding, stability, oligomerization, and subcellular localization (Mitra et al., 2006).



Domain structure and potential post-translational modification sites of human RPRM and RPRML proteins. (Reprint from "The Reprimo Gene Family: A Novel Gene Lineage in Gastric Cancer with Tumor Suppressive Properties" by (Amigo et al., 2018). Retrieved from Int. J. Mol. Sci., 2018;19(7). Doi: 10.3390/ijms19071862. Epub 2018 June 25)

On the other hand, nucleotide sequence homology between *RPRM* and *RPRML* is restricted to their coding sequence and probably have distinct cis- and trans- regulatory elements. However, both genes are located in highly dense CpG islands within the genome (Dunham et al., 2012), suggesting *RPRML* could also be subjected to regulation by DNA methylation.

In summary, *Reprimo* gene family has been poorly studied. The founding member, *RPRM*, is a putative tumor suppressor gene, commonly downregulated in gastric cancer due to methylation of its promoter region. Loss of expression of *RPRM* has been associated with increased invasiveness and poor prognosis of gastric cancer patients. In addition, methylated *RPRM* DNA can be detected in circulation of gastric cancer patients and is a candidate biomarker for gastric cancer detection. *RPRML*, another member of *Reprimo* gene family, has never been studied in humans or in pathology. The high homology it shares with *RPRM* offers a starting point to unravel its biological role.

III. HYPOTHESIS

RPRML acts as a tumor suppressor gene, downregulated by DNA methylation in gastric cancer, and circulates as methylated DNA in gastric cancer patients.

GENERAL OBJECTIVE

To evaluate whether RPRML is silenced in gastric cancer, acting as a tumor suppressor gene regulated by DNA methylation, and circulates as methylated DNA in gastric cancer patients.

SPECIFIC OBJECTIVES

1. To determine RPRML expression and clinical significance in gastric cancer patients
2. To evaluate the role of RPRML on tumor suppression in gastric cancer cell lines
3. To evaluate RPRML expression regulation by DNA methylation in gastric cancer cell lines
4. To detect methylated RPRML on plasma samples from gastric cancer patients

IV. METHODS

In silico analysis

RNAseq and Methylation data from The Cancer Genome Atlas (TCGA) stomach adenocarcinoma (STAD) dataset were downloaded and processed using TCGA-Assembler 2 software package as implemented in R statistical programming language. Differential expression analysis of RNA-seq data was performed using EdgeR after TMM normalization. Statistical significance was adjusted by false discovery rate (BH method). Pairwise analysis of both RNA-seq normalized counts and methylation beta values for single genes between patient-matched tumor and non-tumor adjacent samples were performed using Wilcoxon signed-rank test. Adjusted (edgeR) and unadjusted (pairwise comparison of single genes) P-values < 0.05 were considered statistically significant. Methylation data from gastric cancer cell lines was downloaded from the Broad Institute's Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle/)(Ghandi et al., 2019).

Clinical samples, demographic and pathological data

RPRML protein expression was investigated immunohistochemically in 17 de-identified formalin-fixed and paraffin-embedded (FFPE) stomach whole-tissue sections, retrospectively collected from the archives of the Pathology Department from Hospital Clinico Universidad de Chile (HCUCH). To evaluate the clinical significance of RPRML expression in tumors, RPRML immunohistochemistry (IHC) was performed in a cohort of 90 gastric cancer patients with primary tumor samples arranged in tissue microarrays (TMA) from the Chilean Gastric Cancer Task Forces (FORCE1) (ClinicalTrials.gov identifier: NCT03158571/(FORCE1), Pontificia Universidad Catolica de Chile (PUC) (Owen et al., 2018). Anonymized demographic and pathological information together with molecular profile of PDL1, MLH1, PMS2, MSH2, MSH6, HER2, p16, and p53 proteins were obtained from FORCE1 registry (Cordova-Delgado et al., 2019). Plasma samples for Methylation Specific PCR (MSP) detection of circulating methylated RPRML DNA from 21 gastric cancer patients at Hospital Clinico San Borja Arriaran (FONIS SA06I20019) and 36 altruistic blood donors at Red de Salud UC Blood Bank. For MethyLight assay, plasma samples from 25 GC cases and 64 controls with confirmed pathologic low risk OLGA staging (OLGA 0, I, and II) were obtained from three independent sources: 13 GC cases and 50 controls were prospectively collected between 2018 and 2019 at Hospital Clínico Universidad Católica (HCUC), PUC; 12 GC cases were obtained from the Tumor and Tissue Biobank, HCUCH; and 14 additional controls from the Gastroenterology Unit at Fundacion Arturo Lopez Perez (FALP). Written informed consent was obtained from all participants and a waiver

consent was granted in the case of deceased patients. Ethical approval from Internal Review Board and the Ethics and Scientific Committee at the School of Medicine, PUC was obtained for all supporting projects (FORCE1, FONIS, PREVECAN, GCPL, FONDECYT).

Immunohistochemistry (IHC) and immunocytochemistry

RPRML immunohistochemistry and immunocytochemistry were performed using a polyclonal anti-RPRML antibody (ab204896, Abcam Inc.) and ABC Vectastain R.T.U universal kit (Vector Laboratories Inc). In brief, 4 µm formalin-fixed paraffin-embedded (FFPE) gastric tissue, TMA or cell pellet sections were deparaffinized and re-hydrated through xylens and graded alcohol series. Antigen retrieval was performed by heating in a TintoRetriever Pressure Cooker (BioSB, Inc.) in buffer EDTA pH9 (DAKO, Agilent Technologies) for 20 min. Endogenous peroxidase activity was blocked by treating the slides with 4% hydrogen peroxide solution in methanol. Non-specific protein binding was blocked with Vectastain R.T.U normal horse serum (2.5%) for 10 min. Immunostaining was performed using anti-RPRML antibody (ab204896, Abcam Inc.) in a dilution 1:500 for tissue sections and 1:750 for cell pellets, in Emerald diluent solution (ESBE Scientific, Inc.). Following 1h incubation at room temperature, slides were incubated for 12 min with Vectastain R.T.U biotinylated secondary universal antibody, followed by 12 min incubation with Vectastain Avidin/biotinylated complex (ABC) reagent. Slides were revealed with 3,3 Diaminobenzidine (DAB) substrate chromogen system (DAKO, Agilent Technologies) for 1 min and counterstained with Meyer's Hematoxylin (ScyTek Laboratories). Finally, slides were dehydrated and mounted with a synthetic hydrophobic resin (Thermo Shandon Limited, Thermo Fisher Scientific Inc.). Slides were examined by two pathologists (GCA, TMG), blinded to clinical data. Since quantification of RPRML immunostaining has never been reported, staining was scored semi-quantitatively with 3 different scoring systems(S. W. Kim et al., 2016): (1) An intensity score with 4 levels (0: no staining, 1: weak, 2: moderate, and 3: strong); (2) The proportion of stained cells (range 0 to 1); (3) by calculating the product of the intensity score and the proportion of stained cells. To determine the best scoring system for RPRML, we performed ROC curve analysis to discriminate between a subset of clinical variables, obtaining the best Area Under the Curve (AUC) for scoring system (3) (**Supplementary Table I**). Therefore, RPRML IHC score was defined as (3) the product of Intensity and proportion of stained cells.

Cell culture and transfections

Gastric cancer cell lines (AGS, Hs746T, SNU-1, SNU-5, SNU-16, and KATOIII) were purchased from the American Type Culture Collection (ATCC) and cultured at 37°C and 5% CO_2 in RPMI-1640, DMEM, or IMDM

medium (Hyclone Laboratories, GE Healthcare Life Sciences), supplemented with 10 or 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin according to each cell line specific requirement. Transfection with C-terminal turboGFP tagged pCMV6-RPRML-GFP or pCMV6-AC-GFP empty vector (Origene Technologies Inc.) was performed using Fugene HD (Promega Corp.), according to manufacturer's protocol. Stable transfections were generated by growing transfected cells for 21 days in medium containing G418 antibiotic (500 µg/ml). Surviving cells were sorted by GFP fluorescence in a FACSAria™ II (BDBiosciences) cell sorter (at Fundacion Ciencia y Vida, Santiago, Chile) and then maintained in culture medium containing 250 µg/ml of G418. GFP and RPRML-GFP expression was confirmed by Western Blot analysis and fluorescence microscopy (Carl Zeiss).

RNA isolation and RT-PCR

Total mRNA was isolated using Trizol reagent (Invitrogen, Thermo Fisher Scientific Inc.) according to manufacturer's instructions. RPRML mRNA expression was evaluated through conventional Reverse Transcription Polymerase Chain reaction (RT-PCR). For this purpose, primers were designed using NCBI-Primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in **Supplementary Table II**. Firstly, reverse transcription of 1 µg mRNA was performed with MMLV reverse transcriptase (Promega Corp.) according to manufacturer's recommendations in a final volume of 20µl. Conventional PCR reaction was performed in a final volume of 25 µl containing: 2 µl cDNA, 2 mM MgCl2, 5 mM dNTPs, 0.25 µM of each primer, 5 µl of 5XGreen GoTaq Flexi Buffer (Promega Corp.) and 1 unit of GoTaq G2 DNA polymerase (Promega Corp.). Thermal profile consisted in an initial 5 min incubation at 95°C; followed by 35 cycles of denaturation, annealing and extension periods at 95°C, 57°C , and 72°C respectively (30 sec each), and a final extension period of 10 min at 72°C. RT-PCR products were resolved on 2% agarose gels. RPS13 was used as reference gene.

Protein extraction and Western Blot Analysis

Whole-cell lysates were extracted using RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1 % Triton X-100; and 0.1 % SDS) containing protease and phosphatase Inhibitor Cocktail (Merck KGaA). Total protein content was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.), following manufacturer's protocol. Equal amounts of proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 12%, and then transferred to a polyvinylidene difluoride (PVDF) membrane using a mini Transblot Electrophoretic cell (Bio-Rad Laboratories Inc.). The PVDF membranes were blocked with 5% milk in Tris-Buffered Saline with Tween 20 (TBS-T) buffer for 1h, and

incubated at 4°C overnight with polyclonal antibody anti-RPRML (1:1000, ab204896, Abcam Inc.), previously diluted in TBS-T/3% BSA, anti-turboGFP (1:5000, #PA5-22688, Thermo Fisher Scientific Inc.), anti-DYKDDDDK Tag (1:1000, 9A3, Cell Signaling Technology), B-tubulin (1:2000, #86298, Cell Signaling Technology), or anti-GAPDH (1:2000, Cell Signaling Technology). Subsequently, membranes were washed three times in TBS-T for 10 min each and incubated for 1h at room temperature with the secondary antibody anti-rabbit peroxidase-conjugated (P0448, DAKO, Agilent Technologies), diluted 1:2000 in TBS-T. Finally, membranes washed again and visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

MTS Assay

Wild type, GFP, or RPRML-GFP expressing cells were seeded on 96-well plates at a density of 5×10^3 cells/well and incubated at 37°C and 5% CO2 for 0h, 24h, 48h and 72h. At each time point, MTS colorimetric assay (CellTiter 96® AQueous One Solution MTS assay, Promega Corp.) was performed following the manufacturer's instructions. Briefly, 50 µL of PMS (Phenazine methosulfate) solution was added to 1 ml of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt). Then, 20 µL of MTS + PMS mixture was added to each well and incubated at 37 °C and 5% CO2 for 1h. Metabolic reduction of MTS tetrazolium salt to formazan was measured through Optical Density (OD) at 492 nm wavelength using an EPOCH™ microplate spectrophotometer (Biotek Intruments).

Cell Proliferation and Apoptosis

Cell proliferation and apoptosis were assessed by Ki67 and cleaved-casapase-3 (Cl-caspase-3) immunofluorescence respectively. WT, GFP, or RPRML-GFP stably transfected cells were seeded at a density of 3×10^4 on 12mm cover slides. For proliferation assay, cells were cultured for 48h at 37°C and 5% CO2. For apoptosis assay, cells were cultured for 24h and then starved in culture medium without FBS for 24h. Cells were washed twice with PBS 1X and fixed with Buffered Formalin-Zinc solution (Thermo Fisher Scientific Inc.) at room temperature for 30 min. Cells were permeabilized with 0.1% Triton X-100 in Tris-HCl for 15 min at room temperature, and then incubated with anti-Ki67 primary antibody (1:200 dilution; Cell Signaling Technology) or anti-Cl-caspase-3 (1:200 dilution; D175, Cell Signaling Technology) in Emerald diluent (Merck KGaA) overnight. Then, incubated with Alexa Fluor 546 anti-rabbit IgG (H+L) secondary antibody (1:1000 dilution; Invitrogen, Thermo Fisher Scientific Inc.) for 1 h at room temperature and Hoechst 33342 nucleic staining (1:2000, Invitrogen, Thermo Fisher Scientific Inc.) for 15 min. Cells

were visualized under an epifluorescence microscope (Carl Zeiss) and images were analyzed using Image J software. The percentages of Ki67- or Cl-caspase-3 cells were determined by counting five random fields at 10X magnitudes.

Clonogenic Capacity

Colony formation assay was performed as described by Saavedra et al (Saavedra et al., 2015). Briefly, stably transfected cells were seeded in 12-well plates at a density of 300 cells/well and grown at 37°C for 14 days. Surviving colonies (≥ 50 cells per colony) were counted after fixing and staining with 0.5% crystal violet in 25% methanol/PBS 1X. Digital images were obtained by scanning at high resolution.

Soft Agar Colony Formation Assay

Anchorage-independent cell growth was determined by colony formation in soft agar as described by Borowitz *et al*. (Borowicz et al., 2014). Stably transfected cells at a density of 5×10^3 cells were mixed with 0.3% UltraPure™ LMP Agarose (Invitrogen) in RPMI medium and plated over a solidified layer of 0.6% agarose in RPMI 10% FBS medium in a 12-well plate. Solidification was completed at room temperature for 30 min and incubated at 37°C and 5% CO2 for 21 days. Fresh medium was added every 3 days. At day 21, cells were washed with PBS 1X, fixed with cold 10% Methanol in PBS 1X for 15 min and stained with Crystal Violet 0.0001% in PBS 1X. Colonies larger than 50 µm diameter were counted in each well.

Cell Cycle Analysis

Cell cycle distribution was evaluated by flow cytometric analysis of Propidium Iodide stained cell DNA content. In brief, $1x10^6$ stably transfected cells were grown in 60 mm plates for 24h. Cells were trypsinized and the reaction was ended with 1 ml of RPRMI 10% FBS. Cells were counted and 500,000 cells were placed on falcon tubes, washed with PBS 1X, spin down at 1,000 rpm for 5 min and resuspended in 1 ml PBS 1X. Then, cells were fixed and permeabilized adding dropwise 2.5 ml of cold 95% (v/v) Ethanol in continuous agitation. After fixing the cells overnight at -20°C, the cells were washed with cold PBS 1X and incubated in 300 µl of a solution 20 µg/ml RNAse A in PBS 1X for 1hr at 37°C. Finally, DNA content was analyzed by staining the cells with 2 µl of Propidium Iodide 1mg/ml solution (#P3566, Life Technologies) and flow cytometry (FACSCanto™, BDBiosciences) at Fundacion Ciencia y Vida, Santiago, Chile. Data of 20.000 events/condition was recorded and DNA content frequency histograms (N° of events vs PI intensity) were analyzed using FCS Express DeNovo Software and 2-cycle fitting model.

Inhibition of DNA Methylation

To inhibit DNA methylation *in vitro*, cultured cells were treated with 5-Azacytidine (5-Aza; ab142744, Abcam Biochemicals, Abcam Inc.) as described by Bernal *et al*. (Bernal et al., 2008). In brief, $1x10^6$ AGS, SNU-16, Hs746T, and KATO III cells were seeded and 24h later culture medium was supplemented with 1 or 5μM of 5-Aza. Medium supplemented with 5-Aza was changed every day for 3 days.

DNA isolation and Bisulfite Modification

Genomic DNA from 80% confluent cultured cells was isolated using Wizard SV Genomic DNA purification System (Promega Corp.) following manufacturers recommendations. Plasma DNA from gastric cancer patients and controls was isolated using DNA Blood mini Kit (Qiagen) according to manufacturers instructions, starting from 500 μl plasma and eluting in a final volume of 100 μl. Isolated genomic DNA (1 μg) from cell lines and plasma DNA (20 μl) was then subjected to sodium bisulfite modification using EZ Gold Kit (Zymo Research) according to manufacturer's protocol. Final elution volume was 20μl.

Bisulfite Sequencing

Bisulfite-modified genomic DNA was amplified by PCR using primers listed in **Supplementary Table II**. The PCR profile consisted of an initial denaturation at 95°C for 5 min; followed by 35 cycles of 30 sec at 95°C, 30 sec at Annealing Temperature (Ta, as listed in **Supplementary Table II**), and 1 min at 72°C; and a final extension at 72°C for 5 min. PCR products were sequenced through Macrogen Sequencing Service.

Methylation-Specific PCR (MSP) Assay

For detection of methylated RPRML DNA in plasma samples, specific primers were design using Methprimer 2.0 online platform (http://www.urogene.org/methprimer2/) and are listed in **Supplementary Table II**. MSP reaction was performed in a final volume of 25 μl containing 2 μl bisulfite-converted DNA, 2mM MgCl2, 5mM dNTPs, 0.25μM of each primer, 5μl of 5XGreen GoTaq Flexi Buffer (Promega Corp.), 1 unit of GoTaq G2 DNA polymerase (Promega Corp.), and 1μM Betaine (Merck KGaA). Thermal cycling conditions consisted in an initial denaturation step at 95°C for 5 min; followed by 35 cycles of denaturation, annealing and extension periods at 95°C, Annealing Temperature (Ta, as listed in **Supplementary Table II**), and 72°C respectively (30 sec each); and a final extension step at 72˚C for 10 min. Normal peripheral blood cells (PBCs) DNA (unmethylated) and artificially methylated DNA were used as negative and positive controls, respectively.

MethyLight assay

Bisulfite-modified plasma DNA was amplified by Taqman based quantitative PCR (Eads et al., 2000) using a Rotor-Gene Q5 plex Platform (Qiagen). RPRML locus specific amplification was performed using primers flanking a 142bp target region within RPRML 5'UTR (+11 to +152 from Transcription start site), and an oligonucleotide probe with a 5' fluorescent reporter dye (FAM) and a 3' quencher (TAMRA) as listed in Supplementary Table **II**. Amplification of a methylation-independent sequence from MYOD1 gene was used as a control of DNA input as described elsewhere (Eads et al., 1999). MethyLight reaction was performed using 4 µl of bisulfite-modified plasma DNA, 1X of LightCycler FastStart DNA Master Hybprobe (Roche), 0.6 µM of each primer, and 0.2 µM of oligonucleotide probe. Thermal cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles at 95°C for 5 sec and 60°C for 55 sec. For absolute quantification, a standard curve was prepared by serial dilutions of a synthetic double stranded DNA fragment (Integrated DNA Technologies, Inc.) starting at 1 ng/µl. A reference dilution was included in each plate to normalize between plates. Ct values obtained from plasma samples were subsequently interpolated on the standard curve to determine the number of DNA copies/µl plasma.

Statistical analysis

To evaluate the difference in RPRML expression between matched-pairs of tumors and NTAM from HCUCH FFPE stomach whole-tissue sections (IHC score), Wilcoxon matched-pairs signed-rank test was used. To assess the clinical significance of RPRML expression, IHC scores from tumors in FORCE1 study were treated as a continuous variable and were associated with clinicopathologic variables by Wilcoxon sum-rank test (two sided) or Welch's unequal variances t-test for association between two categories, and Kruskal-Wallis test for three or more categories. In addition, univariate and multivariate Cox proportional hazards model was used to evaluate the effect of RPRML IHC score on global survival. For *in vitro* functional assays, differences between groups were assessed by Kruskal-Wallis and Dunn's Multiple Comparison Test. To analyze differences in MSP detection of methylated RPRML DNA between plasma samples from gastric cancer patients and blood bank donors, Mann-Whitney U test was performed. Finally, to evaluate de ability of circulating methylated RPRML DNA to discriminate gastric cancer patients from low risk OLGA controls by MethyLight assay we performed Receiver Operating Curve (ROC) analysis, and selected the best cutoff value based on the maximization of the Youden Index = Sensitivity + Specificity – 1 (Youden, 1950). In all cases, a p-value <0.05 was considered significant.

V. RESULTS

1. RPRML expression and clinical significance in gastric cancer.

To determine RPRML expression in gastric cancer, we analyzed RNAseq data from 32 matched-pairs of stomach adenocarcinoma and non-tumor adjacent mucosa (NTAM) tissues from the TCGA STAD dataset [Cancer Genome Atlas Research Network, 2014, 25079317]. As shown in **Figure 1A**, RPRML transcript expression was significantly downregulated in tumor tissues compared to NTAM (p=0.01681). To confirm this observation, we assessed RPRML protein expression by immunohistochemical (IHC) staining in 17 paired stomach adenocarcinoma and NTAM tissues from the archives of the HCUCH. Specificity for RPRML protein was tested by Western Blot analysis as shown in **Supplementary Figure 1**. As shown in **Figure 1B**, RPRML IHC staining was predominantly localized in the cytoplasm of foveolar and glandular epithelial cells in gastric mucosa and was reduced in tumor cells. Slides were examined by a single pathologist (GCA) and scored by combining staining intensity and extent as described in Methods section (RPRML IHC score). In NTAM tissues, median RPRML IHC score was 1.5 (Interquartil Range, IQR: 1.0 - 1.5) and in tumors, median was 0.5 (IQR: 0.1 - 1.0) **(Figure 1C)**. Paired differential analysis showed a statistically significant downregulation in tumors (p=0.001), consistent with RNA-seq data analysis from the TCGA.

To evaluate the clinical significance of RPRML loss of expression in gastric cancer, RPRML IHC protein staining was assessed in a cohort of 90 gastric cancer patients with primary tumor tissue samples from FORCE1 Study (Cordova-Delgado et al., 2019; Owen et al., 2018). RPRML IHC staining was examined by two pathologists (GCA and TMG), and scores were correlated with clinicopathologic data, overall survival (OS), and status of relevant molecular markers in gastric cancer. Median RPRML IHC score was 0.0237 (IQR: 0 – 0.0240) with a skewed distribution to the left. For correlation analysis, RPRML IHC score was treated as a continuous variable. As shown in **Table I**, no statistically significant association with clinicopathological features were found. However, RPRML IHC score correlated positively and significantly with overexpression of Erb-B2 receptor tyrosine kinase 2 (Her2) (p=0.036), a proto-oncogene frequently amplified in gastric cancer (**Table II**).

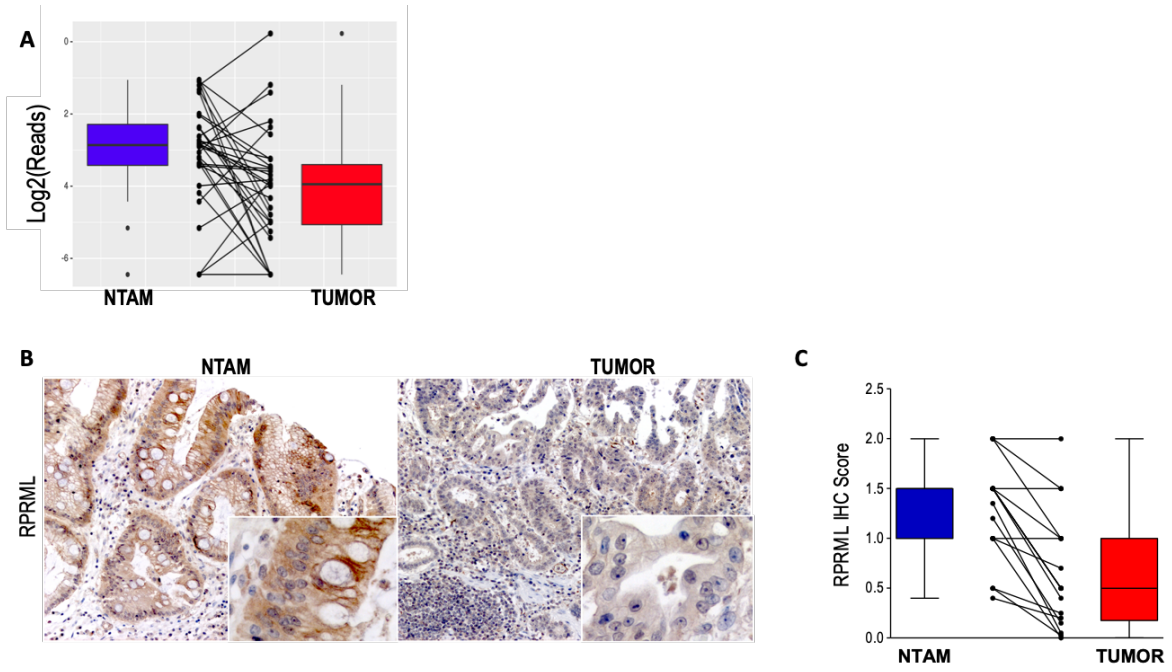


Figure 1. RPRML expression is downregulated in gastric cancer. A. RPRML transcript expression levels in 32 matched-pairs of tumor and non-tumor adjacent mucosa (NTAM) from the stomach adenocarcinoma (STAD) dataset in The Cancer Genome Atlas (TCGA) repository. Box plots (median ± interquartil range) and aligned dot plots of log2 normalized read counts per sample. Differential analysis was performed by Wilcoxon matched-pairs signed rank test (p= 0.0168). **B.** Representative images of RPRML protein immunohistochemical (IHC) staining in 17 matched-pairs of tumor and non-tumor adjacent mucosa (NTAM) samples from gastric cancer patients. Magnification at 200X and 400X for inserts. **C.** Differential RPRML protein expression in paired tumor and NTAM samples from gastric cancer patients. Box plots (median ± interquartil range) and aligned dot plots of RPRML IHC score. Statistical analysis was performed using Wilcoxon matched-pairs signed-rank test (p= 0.001).

Table I. Association of RPRML protein expression with clinicopathologic features of gastric cancer patients

Characteristic	N	%	RPRML IHC Score	
			Median	*P* Value
Sex				
Male	59	65.56	0.015	0.15[a]
Female	31	34.44	0.0375	
Age				
<60	35	39.33	0.0125	0.98[a]
>60	54	60.67	0.0294	
Lauren histological type				
Intestinal	28	38.89	0.0375	0.56[c]
Diffuse	31	43.06	0.0313	
Mixed	13	18.06	0.0688	
Signet-ring cell presence				
No	57	63.33	0.0125	0.16[a]
Yes	33	36.67	0.0625	
Localization				
Proximal	18	20.93	0.0113	
Medial	37	43.02	0.0625	0.41[c]
Distal	27	31.4	0.0113	
Multiple	4	4.65	0.2875	
Lymph node Metastasis				
No	17	22.08	0.0625	0.18[b]
Yes	60	77.92	0.0144	
Peritoneal involvement				
No	88	97.78	0.0294	0.61[a]
Yes	2	2.22	0.0106	
Hepatic Metastasis				
No	85	97.7	0.0313	0.43[a]
Yes	2	2.3	0.2575	
TNM Stage				
I-II	32	35.56	0.0138	0.53[a]
III-IV	58	64.44	0.0343	

[a] Wilcoxon sum rank test, [b] Welch's unequal variance t-test, [c] Kruskall-Wallis Test.

Table II. Correlation of RPRML expression with molecular markers in primary tissues

Molecular marker	N (%)	RPRML IHC Score	
		Median	*P* Value
PD-L1			
Negative	64	0.0531	0.18[b]
Positive	26	0.0031	
MSI			
Negative	77	0.0375	0.41[a]
Positive	13	0.0138	
HER2			
Negative	79	0.0125	**0.036[b]**
Positive	11	0.3375	
p53			
Negative	52	0.0169	0.88[a]
Positive	38	0.0375	
p16			
Lost	32	0.0025	0.38[b]
Normal	55	0.0500	
E-cadherin			
Lost	15	0.0100	0.66[a]
Normal	74	0.0344	

[a] *Wilcoxon sum rank test,* [b] *Welch's unequal variance t-test*

In addition, we investigated the association of RPRML protein expression with OS (**Table III**). Univariate Cox proportional hazard model did not show statistically significant association (HR 0.30, 95%CI:0.07-1.27, *p*=0.102). However, after adjusting by age, gender and TNM stage in the multivariate model, RPRML expression significantly associated with OS (HR 0.17, 95%CI:0.04-0.78, *p*=0.022), decreasing the hazard of death by 83% (as 1 - 0.17 = 0.83) for every unitary increase in the IHC score. Or in other words, by every unit decrease in RPRML IHC score, survival probability decreased 5.9 times (as the inverse of HR 1/0.17) at any time point. To further evaluate the relationship between RPRML IHC score and TNM stage, we modelled Cox regression by subgroups: early- (TNM stages I-II) and advanced-stage (TNM stages III-IV) disease. Results in **Table IV** indicate RPRML expression was a significant prognostic factor for advanced-stage gastric cancer patients (HR 0.07, 95%CI: 0.01-0.46, *p*=0.005) but not for early-stage patients (HR 3.26, 95%CI: 0.30-35.84, *p*=0.334).

To gauge the impact of RPRML expression in OS, all advanced gastric cases were stratified into high- and low- RPRML expression groups using an optimal cut-off value for RPRML IHC Score determined by ROC curve analysis (**Supplementary Figure 2).** By this approach, survival rates at 2- and 5- years of the low expression group were half that of the high expression group (2-year survival= 40.0 vs. 81.3 months, respectively; and 5-year survival= 17.0 vs. 53.5 months, respectively) (**Figure 2).** Overall comparison

showed that the low-expression group had significantly worse prognosis compared to high- expression group (p=0.00051, Log-Rank test).

Table III. Uni- and Multivariate associations between clinicopathologic features and overall survival

		Mean (SD) or N (%)	Univariate Analysis		Multivariate Analysis	
			Hazard Ratio	*P*-value	Hazard Ratio	*P*-value
Age		62.5 (13.4)	1.02 (1.00-1.04)	0.122	1.01 (0.99-1.04)	0.165
Gender						
	Male	59 (65.6)	Reference		Reference	
	Female	31 (34.4)	1.34 (0.76-2.37)	0.313	1.15 (0.65-2.05)	0.631
Stage						
	I-II	32 (35.6)	Reference		Reference	
	III-IV	58 (64.4)	3.38 (1.69-6.78)	0.001	3.76 (1.86-7.62)	**<0.001**
RPRML IHC-score		0.1 (0.2)	0.30 (0.07-1.27)	0.102	0.17 (0.04-0.78)	**0.022**

Table IV. Association analysis between RPRML expression and overall survival by TNM stage subgroup

	Subgroup	Hazard Ratio	*P*-value
RPRML IHC Score	**Stage I-II**	3.26 (0.30-35.84)	0.334
	Stage III-IV	0.07 (0.01-0.46)	**0.005**

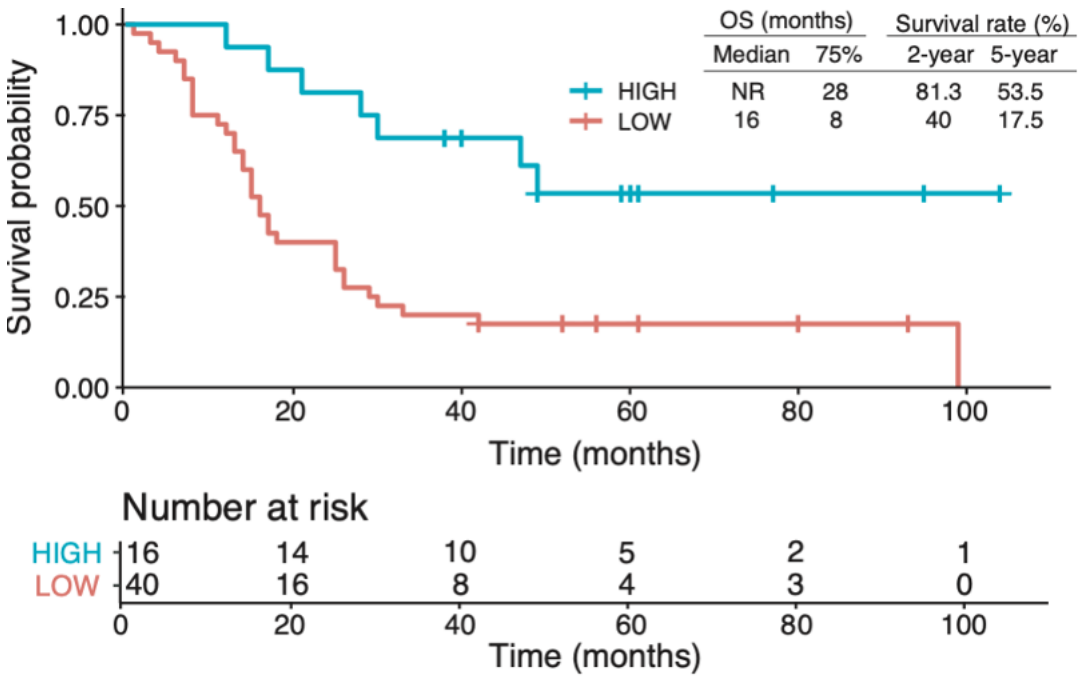


Figure 2. Overall survival (OS) according to the expression levels of RPRML in gastric cancer. Kaplan-Meier curves showing OS probability of patients with high versus low RPRML expression (cut-off IHC score = 0.162) among 56 advanced (TNM stage III and IV) gastric cancers). Median and 75% percentile OS times, and two- and five-year survival rates are shown above. Overall comparison between groups was evaluated by Log-Rank test (p=0.00051). In the lower panel, the number at risk represents the number of patients living at each time point. Please see **Supplementary Table III** for a detailed count of the number of deaths at each time interval.

2. Role of RPRML on tumor suppression *in vitro*.

In view of the strong association we found between RPRML expression and OS, we proceeded to investigate cell biological characteristics that could lead to tumor progression when overexpressing RPRML i*n vitro*.

2.1 Characterization of RPRML expression in gastric cancer cell lines

To select an appropriate *in vitro* model for studying RPRML effect on tumor cell biology, we first characterized RPRML expression in a panel of six gastric cancer cell lines (i.e. AGS, SNU-1, SNU-5, SNU-16, KATO III and Hs746T) by RT-PCR and Western Blot analysis. As shown in **Figure 3A**, we detected RPRML mRNA in SNU-1 and SNU-5 cell lines. However, we were unable to detect endogenous RPRML protein by Western Blot analysis in these cell lines **(Figure 3B)**. Therefore, we assessed RPRML endogenous protein expression by immunocytochemistry, confirming positive protein expression in SNU-1 and SNU-5 cell lines and negative in AGS cell line **(Figure 3C).**

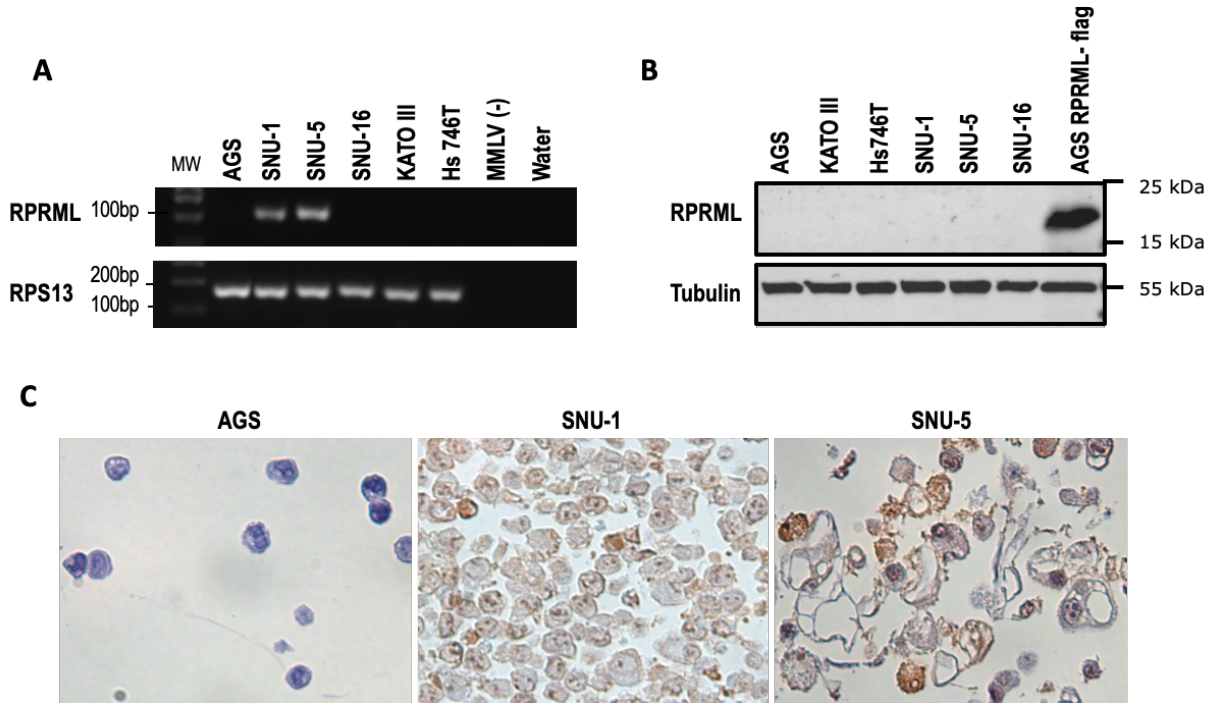


Figure 3. Characterization of RPRML expression in gastric cancer gastric cancer cell lines. A. Detection of RPRML mRNA in gastric cancer cell lines assessed by conventional RT-PCR. MW: molecular weight DNA ladder, MMLV(-): Reverse transcription negative control. **B.** Western Blot analysis of RPRML in wild type gastric cancer cell lines. Protein lysate from AGS cells transfected with pCMV6-RPRML-DDK flag was used as a positive control (last lane). **C.** Immunocytochemistry of RPRML in AGS, SNU-1 and SNU-5 cell pellets.

Since this is the first report of RPRML protein detection, we also assessed RPRML coding potential by analysis of public data of ribosome profiling (Ribo-seq) experiments. This technique, based on sequencing of ribosome protected mRNA fragments, has emerged in recent years as a powerful and accurate estimation of genome-wide protein synthesis (Michel et al., 2014). Using GWIPS-viz web tool (http://gwips.ucc.ie) we found that elongating and initiating ribosome read counts were consistent with the annotated coding region of the RPRML gene (**Supplementary Figure 3**).

2.2 Evaluation of RPRML effect on tumor cell biology *In vitro*

To evaluate the effect of RPRML on tumor suppressive features *in vitro*, AGS cells were stably transfected with pCMV6-GFP (GFP) or pCMV6-RPRML-GFP (RPRML-GFP) by G418 antibiotic selection. GFP-positive cells were recovered by fluorescence- activated cell sorting (FACS) for further analysis. GFP and RPRML-GFP expression were confirmed by florescence microscopy (**Figure 4A)** and Western Blot analysis **(Figure 4B).**

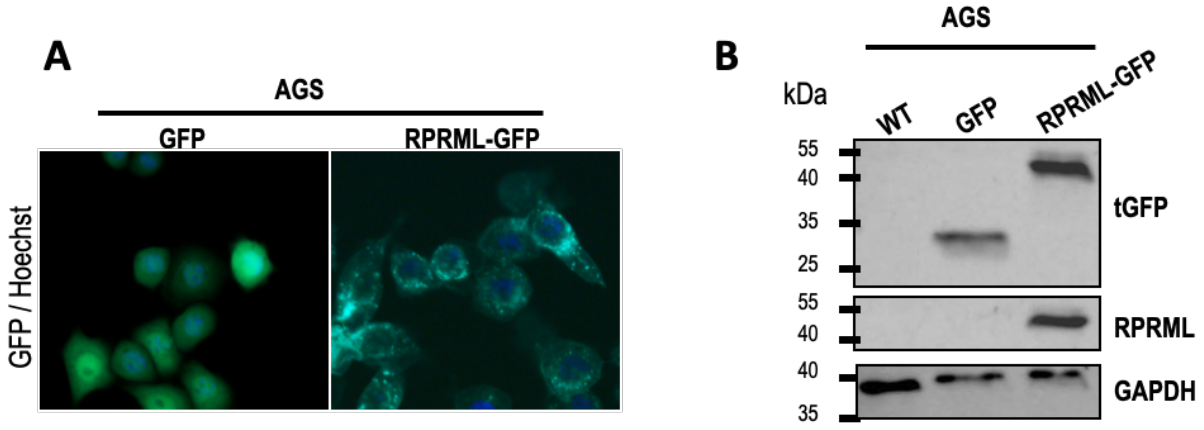


Figure 4. Stable transfection of RPRML-GFP in AGS cell line. A. Representative images of GFP and RPRML-GFP overexpressing AGS cells. Live cells were stained with Hoechst and analyzed by fluorescence microscopy at magnification 40X. **B.** Western Blot analysis of wild type (WT), GFP, and RPRML-GFP overexpressing AGS cells using anti-turbo GFP (tGFP) or anti-RPRML antibodies. GAPDH was used as a loading control.

After establishing RPRML overexpression model, we addressed the effect of RPRML on cell proliferation/ viability indirectly through quantification of the metabolic reduction of MTS tetrazolium salt to formazan (MTS assay). As shown in **figure 5A**, RPRML overexpression significantly reduced cell proliferation/viability in AGS cells 24, 48 and 72h after seeding. Next, we evaluated the effect of RPRML overexpression on cell proliferation by Ki67 immunofluorescence. RPRML overexpression significantly reduced the percentage of Ki67-positive cells by nearly 50% in RPRML-GPF expressing cells compared to wild type (p<0.05) and GFP-overexpressing cells (p<0.05) **(Figure 5 Band C)**. In a similar manner, we explored the role of RPRML on apoptosis through immunofluorescence of Cl-caspase-3 (**Figure D and E**). Apoptosis was induced by serum starvation for 24h, however, it was not affected by RPRML overexpression.

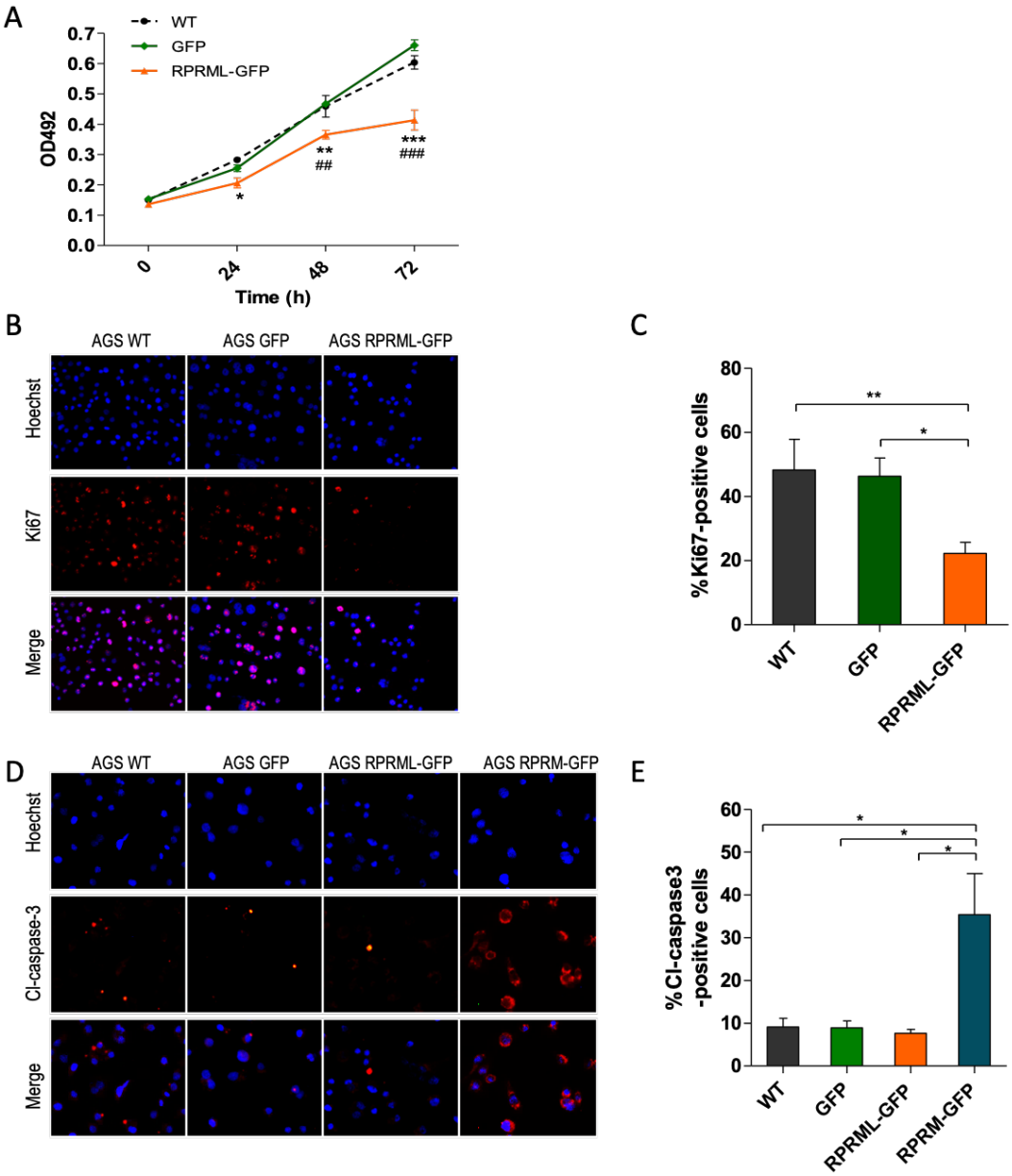


Figure 5. RPRML overexpression decreases cell proliferation in AGS cell line. A. Cell proliferation/viability of wild type (WT), GFP, and RPRML-GFP overexpressing AGS cells was evaluated by MTS assay at 0h, 24h, 48h and 72h (n=3). Statistical Analysis: Two-way ANOVA and post Bonferroni Test. *:WT vs RPRML-GFP, #: GFP vs RPRML-GFP. $p<0.05$ (*, #), $p<0.01$ (**, ##) and $p<0.0001$ (***, ###). **B.** Representative images of Ki67 immunofluorescence (red) and Hoechst staining (blue) in WT, GFP, and RPRML-GFP overexpressing AGS cells, 48h after seeding**. C.** Percentage of Ki67 positive cells. Five random fields at 10X magnitude were quantified using Image J software. Results represent the means of three independent experiments, bars indicate SEM. Statistical analysis: Kruskal-Wallis followed by Dunn's Multiple Comparison Test (*$p<0.05$). **D.** Representative images of cleaved-caspase-3 (Cl-caspase-3) immunofluorescence (red) and Hoechst staining (blue). WT, GFP, or RPRML-GFP overexpressing AGS cells were cultured for 24h in complete medium and then starved in culture medium without FBS for 24h to induce apoptosis**.** AGS cells overexpressing RPRM and starved without FBS were used as a positive control. **E.** Percentage of Cl-caspase-3 positive cells. Quantification of five random fields at 10X magnitude. Results represent the means of three independent experiments, bars indicate SEM. Statistical analysis: Kruskal-Wallis followed by Dunn's Multiple Comparison Test.

In addition, we evaluated the effect of RPRML overexpression on cell cycle progression through the measurement of cellular DNA content stained with Propidium Iodide (PI) by Flow cytometry. As shown in **Figure 6,** RPRML overexpression significantly decreased the percentage of cells in G0/G1 phase and increased the percentage of cells in G2/M phase compared to control GFP-expressing cells.

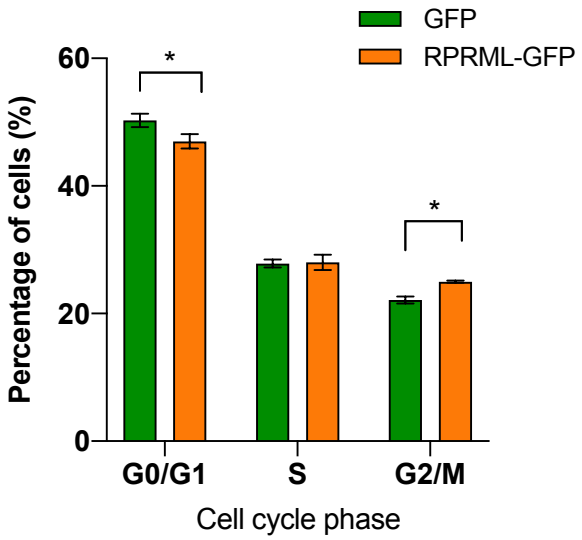


Figure 6. Effect of RPRML overexpression on cell cycle progression. DNA content was analyzed by flow cytometry of PI stained cells. Data of 20.000 events/condition was recorded and frequency histograms were analyzed using FCS Express DeNovo Software. Results represent the mean percentage of cells distributed in G0/G1, S, and G/M phases of three independent experiments. Statistical analysis was performed by multiple t-test with the assumption of consistent SD for each phase (*p<0.05). Frequency histograms for the three independent experiments are presented in **Supplementary Figure 4**.

To further explore the role of RPRML on cellular malignant transformation we evaluated the effect of RPRML overexpression on clonogenic capacity and anchorage-independent growth by colony formation and soft-agar assays in AGS cells. As shown in **Figure 7**, RPRML overexpression significantly inhibited both colony formation (p=0.007) and soft-agar anchorage-independent growth (p=0.001) .

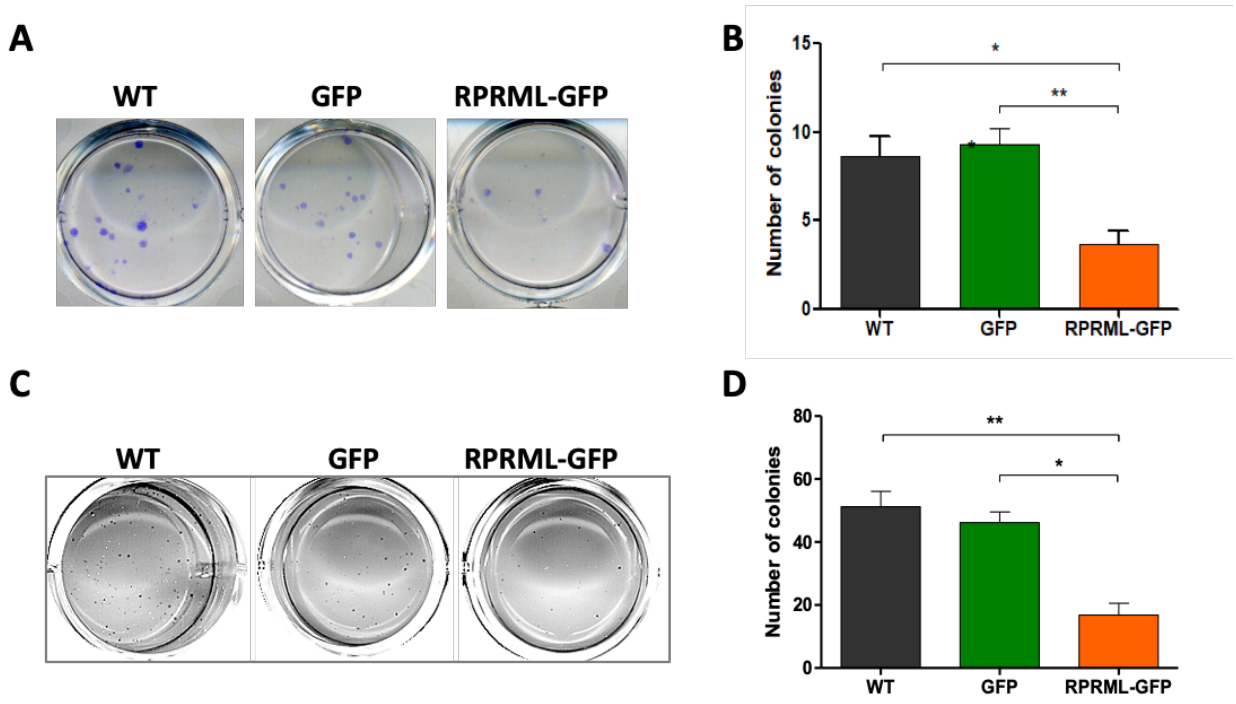


Figure 6. RPRML overexpression reduces clonogenic capacity and anchorage-independent growth of AGS gastric cancer cells. A. Representative images of colony formation assay in WT, GFP and RPRML-GFP overexpressing AGS cells. **B.** Quantification of colony formation assay. Results represent the mean of three independent assays and SEM. Statistical Analysis: Kruskal-Wallis (p=0.007) followed by Dunn's Multiple Comparison Test (**p<0.01, *p<0.05). **C.** Representative images of soft-agar anchorage-independent colony formation assay in WT, GFP, or RPRML-GFP overexpressing cells. **D**. Quantification of soft agar colony formation assay. Colonies > 50 µm were counted. Results represent the mean of three independent assays and SEM. Statistical Analysis: Kruskal-Wallis (p=0.001) followed by Dunn's Multiple Comparison Test (**p<0.01, *p<0.05).

3. RPRML expression regulation by DNA methylation in gastric cancer cell lines

Since RPRML expression was downregulated in gastric cancer, we evaluated whether this could be due to genetic alterations by assessing the frequency of *RPRML* genetic alterations in the TCGA dataset(Cancer Genome Atlas Research Network, 2014). However, only 1% of 393 gastric cancer cases carried a genetic alteration in *RPRML* gene, and these alterations were mostly copy number amplifications. Moreover, given that *RPRML* gene is located within a dense CpG island, we hypothesized that RPRML expression could be regulated by DNA methylation.

To evaluate whether DNA methylation affects RPRML expression, we evaluated whether demethylation of DNA restores RPRML transcript expression. Gastric cancer cell lines with undetectable RPRML transcript expression (as characterized in **Figure 3**) were treated with the well-established

methylation inhibitor 5-Azacytidine (5-Aza) and RPRML expression was evaluated by conventional RT-PCR. As shown in **Figure 8A,** treatment with 1µM of 5-Aza restored RPRML transcript expression in SNU16, and Hs746T cell lines, but not in AGS cell line. To evaluate if re-expression was dose-dependent in AGS cells, we increased the concentration of 5-Aza to 5uM (data not shown). However, RPRML transcription was not restored. In addition, we explored re-expression of RPRML protein after treatment with 5-Aza in SNU-16 cell line, however no protein product could be detected by Western Blot analysis (**Supplementary Figure 5).**

Several studies analyzing large-scale data have reported that the strongest correlation between DNA methylation and gene expression occurs within the nearest region surrounding the transcription start site (TSS) of a gene (Spainhour et al., 2019; Wagner et al., 2014). We assessed the DNA methylation status of a region +111 to +248 relative to the TSS of *RPRML* gene by direct bisulfite sequencing analysis of AGS, Hs746T, and SNU-16 cell lines, together with SNU-16 treated with 5-Aza inhibitor (**Figure 8B**). Results from this analysis indicated that all CpGs analyzed were completely methylated in AGS, Hs746T and SNU-16 cell lines, while most CpGs were demethylated in SNU-16 cell line treated with 5-Aza inhibitor.

Moreover, the recent release of The Broad Institute's Cancer Cell Line Encyclopedia (CCLE) (Ghandi et al., 2019), a large -scale characterization of 1,072 human cancer cell lines, allowed as to assess individual CpG methylation status of *RPRML* gene (region -781 to +435 relative to TSS), from the available gastric cancer cell lines: AGS, Hs746T, and SNU-5 (**Supplementary Figure 6**). SNU-5 cell line, which was positive for RPRML mRNA expression in our previous characterization (**Figure 3**), displayed low Beta values (range 0-0.1) in all CpG sites analyzed. Whereas, most CpG sites from AGS and Hs746T cells, with undetectable RPRML mRNA expression, ranged in the upper levels of Beta values (>0.7). In addition, we interrogated DNA methylation status from the TCGA STAD dataset (Cancer Genome Atlas Research Network, 2014) in 59 matched-pairs of stomach adenocarcinoma and non-tumor adjacent mucosa (NTAM) (**Supplementary Figure 7**). Beta values for probe cg08631151 (-300 relative to RPRML TSS) were significantly higher in tumors compared to NTAM ($p=0.006$). Furthermore, a significant correlation between RPRML RNAseq transcript levels and *RPRML* methylation status (cg08631151) was observed for all stomach adenocarcinomas in the TCGA STAD dataset ($r=-0.119$, $p<0.05$).

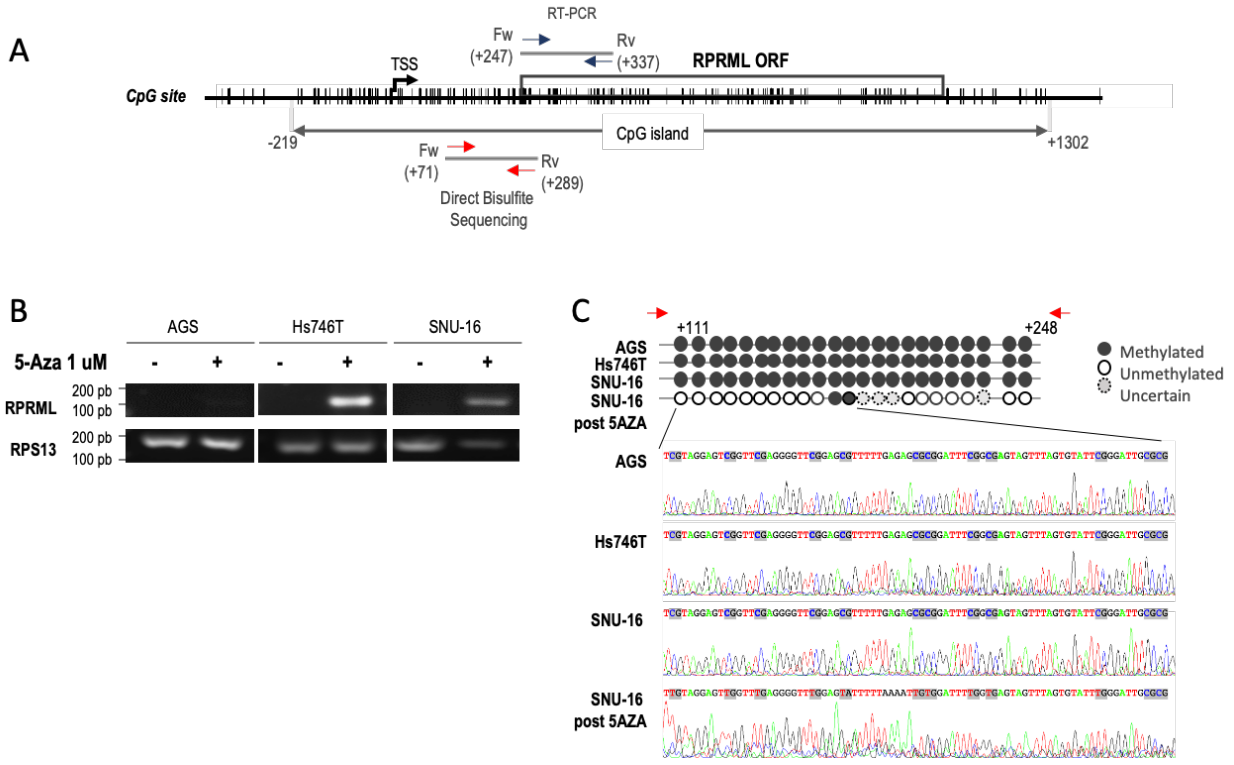


Figure 8. RPRML gene structure, methylation status and transcript expression in gastric cancer cell lines after 5-Azacytidine (5-Aza) assay. A. Schematic representation of RPRML gene structure, Open Reading Frame (ORF), and CpG sites (vertical lines). A dense CpG island is located between nucleotides -219 and +1302 relative to de transcription start site (TSS). The region analyzed within *RPRML* gene by direct bisulfite sequencing is located between nucleotides +71 to +289 relative to the TSS. RT-PCR primers are located between +247 and +337 nucleotides. **B.** RPRML mRNA expression by RT-PCR in three gastric cancer cell lines treated with 1 µM of 5-Aza. **C.** Schematic representation of direct bisulfite sequencing results in three gastric cancer cell lines (AGS, Hs746T, SNU-16). Individual CpG methylation status is represented by black (methylated) and white (unmethylated) circles. Representative DNA sequence electropherograms are shown. In the lower panel, the result of direct bisulfite sequencing after treatment with 1µM of the DNA methylation inhibitor 5-azacytidine (5-Aza) is shown.

4. Detection of methylated RPRML DNA on plasma samples from gastric cancer patients

Our previous results suggested that RPRML expression is consistently downregulated in gastric cancer, and that this downregulation is mediated by DNA methylation, we explored whether methylated *RPRML* DNA could be detected in plasma samples from gastric cancer patients for future potential biomarker studies. To this end, we designed specific primers directed to bisulfite-modified DNA near the TSS region of *RPRML* gene and assessed *RPRML* DNA methylation in plasma samples from 21 gastric cancer patients

and 36 non-consultant blood donors (controls) by MSP assay (**Figure 9**). Circulating methylated *RPRML* DNA was detected in 43% (9/21) of gastric cancer patients but only in 8% (3/36) of controls(p=0.002).

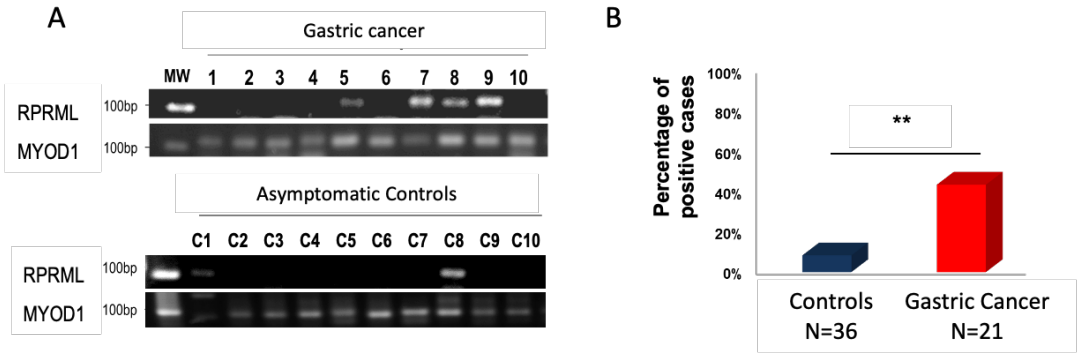


Figure 9. Detection of *RPRML* methylation in plasma DNA from gastric cancer patients and controls by MSP assay. **A.** Representative image of an MSP assay for methylated *RPRML* DNA in plasma samples. A methylation-independent sequence from *MYOD1* gene was used as a control of DNA input. **B.** Graphic representation of the percentage of gastric cancer patients and non-consultant blood donors (controls) with positive detection of methylated *RPRML* DNA in plasma samples. Statistical Analysis: Mann-Whitney U test (**p=0.002).

This result suggests that circulating methylated *RPRML* DNA can be detected in plasma samples and could potentially discriminate between healthy or gastric cancer patients. However, since MSP assay allows detection of only a few CpGs and does not allow quantitative detection of methylated DNA, it is well recognized to have poor sensitivity (Hernández et al., 2013). Therefore, we optimized *RPRML* DNA methylation detection to a more sensitive and high-throughput strategy, based on Taqman quantitative PCR(Eads et al., 2000) termed MethyLight. We used the same primer set used for MSP assay and added a Taqman probe to cover 10 CpGs from a 142bp target region. We generated a standard curve of serial dilutions from a synthetic double stranded DNA fragment of known concentration for subsequent interpolation of Ct values and absolute quantification. By this approach, we quantified methylated *RPRML* DNA in plasma samples from 25 gastric cancer patients and 64 controls with confirmed pathologic low risk OLGA staging (OLGA 0, I, II). Results were analyzed by ROC curve analysis obtaining an AUC of 0.729 (95%CI: 0.599-0.860, *p*=0.001) (**Figure 10**). The cutoff point that maximized the sensitivity and specificity for the detection of gastric cancer was 19.0 copies/mL of plasma. Using this cutoff value, we detected *RPRML* methylation in 14 of 25 gastric cancer patients, obtaining a sensitivity of 56.0% (95%CI:34.93-75.60). Eight of the 64 controls tested positive for *RPRML* methylation obtaining a specificity of 87.5% (95%CI: 76.85-94.45). Positive predictive value was 63.64% (95%CI: 45.62- 78.50) and Negative Predictive

Value 83.58% (95%CI: 68.69-88.89). Positive Likelihood ratio (LR+) was 4.48 (95%CI: 2.15 to 9.35) and Negative Likelihood Ratio (LR-) 0.50 (95%CI: 0.32 to 0.79). Odds ratio was 8.91 (95% CI: 3.02 - 26.31, p= 0.0001).

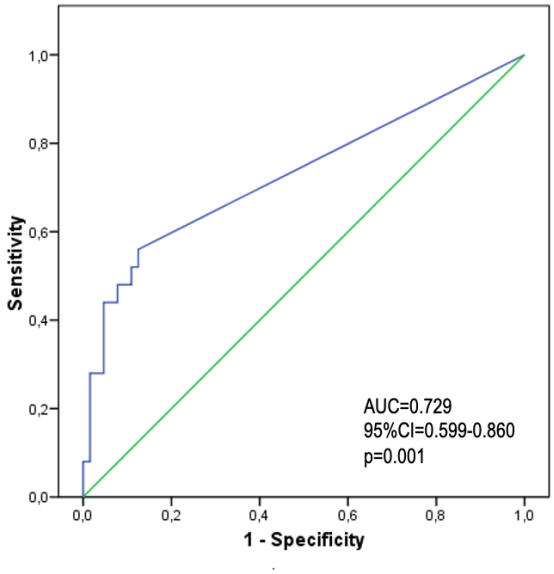


Figure 10. Receiver Operating Characteristic (ROC) analysis of *RPRML* methylated DNA as a biomarker for detection of gastric cancer. Plasma samples from 25 gastric cancer cases and 64 controls with confirmed diagnosis of low risk OLGA (0-II) were analyzed by MethyLight assay. Blue line represents ROC curve for methylated *RPRML* DNA, Green line represents no discrimination. AUC of 0.729 (95%CI: 0.599-0.860, p=0.001).

VI. DISCUSSION

Gastric cancer develops and progress as a consequence of accumulative genetic and epigenetic alterations with tumor suppressive and oncogenic capacity. A broader understanding of individual molecular inter-players during malignant transformation is critical to develop opportune diagnostic and therapeutic strategies. The purpose of this study was to evaluate whether the hitherto uncharacterized member of the *Reprimo* gene family -*RPRML*- is silenced in gastric cancer, has tumor suppressor properties and whether regulation by DNA methylation may serve as potential non-invasive biomarker of gastric cancer.

The results of this investigation indicate that *RPRML* expression is consistently downregulated in gastric cancer. This downregulation was observed both at the protein level, in our set of cases, and at the transcription level, in the STAD-TCGA dataset. Over recent years, there has been a rapid expansion in the number of identified genes differentially expressed in gastric cancer (Vecchi et al., 2007; W. Wang et al., 2020). According to the classic view of Knudson's two hit hypothesis, bi-allelic inactivation of tumor suppressor genes was necessary to drive carcinogenesis (Knudson & Meadows, 1976). By now, there is a wealth of evidence showing that partial loss of multiple genes with tumor suppressive capacity can also contribute to the pathogenesis of cancer (Berger et al., 2011; L. H. Wang et al., 2019). Moreover, dysregulated genes may act as biomarkers of disease, prognosis or response to therapy, and even as potential therapeutic targets (Satherley et al., 2015). Therefore, it is necessary to study the significance of RPRML downregulation in gastric cancer.

The clinical and pathological implications of RPRML downregulation in gastric cancer were evaluated through association analysis between RPRML protein expression and clinicopathological characteristics along with follow-up data. Although RPRML IHC score distributed equally among clinicopathologic features such as sex, age, Lauren histological type, tumor localization, and TNM stage; remarkably, low RPRML protein expression was associated with worse prognosis in advanced-stage of disease. Of note, several authors have proposed that the proof of principle that a gene is a driver of cancer is that it has an impact on survival (Bailey et al., 2018; H. Kim & Kim, 2018; Rafii et al., 2014). Thus, our results suggest that downregulation of RPRML is a risk factor for poor prognosis in advanced-stage disease and might contribute to drive the progression of gastric cancer.

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Currently, TNM stage is the most important prognostic factor of gastric cancer (Amin et al., 2017). Despite the fact that RPRML IHC score is not associated with OS in early stage disease, this group of patients usually presents good prognosis. On the contrary, more than 80% of all cases are diagnosed in advanced TNM stages when they have limited treatment options, and often present different clinical outcomes and response to treatments (Price et al., 2012). In this context, previous research regarding the implications of human epidermal growth factor receptor 2 (HER2) overexpression in the prognosis of gastric cancer was key for the development of standard management of advanced disease (Palle et al., 2020). Today, molecular targeted therapy against HER2 positive tumors (ie, trastuzumab) in combination with chemotherapy is a standard of care, with significant survival benefit for HER2 positive gastric cancer (Bang et al., 2010). However, HER2 positivity occurs only in in 15-20% of gastric cancer and OS of advanced disease remains very poor (Palle et al., 2020). Our findings of RPRML as a significant prognostic factor for advanced gastric cancer may potentially help to identify patients at increased risk of death and may be a potential actionable target for advanced gastric cancer, as has been concept proofed for its homologous, RPRM, using CRISPR technology (Garcia-Bloj et al., 2016). Nevertheless, validation of RPRML low expression as a risk factor for poor prognosis in gastric cancer in a second cohort is warranted.

Conversely, we found a positive and significant association between RPRML expression and HER2 overexpression. We speculate that this association could be due to the close location of both genes at chromosomal location 17q (q12 and q21, respectively); a locus commonly affected by chromosome rearrangements and segmental amplifications in multiple tumors (El–Rifai et al., 2001; Mahlamäki et al., 2002; Marotta et al., 2017). More research would be needed to clarify if this is the case of a passenger co-amplification or if there is any relationship between the expression of both genes, however, it is beyond the scope of this study. Of note, OS between HER2 positive and negative patients was not significantly different ($p=0.62$, Log-rank test) and did not affect RPRML prognostic value.

However, the results presented herein are limited by the lack of available data on the different treatment interventions received by each patient, which could have affected OS of RPRML low- and high expression groups. In addition, RPRML IHC analysis should be interpreted with caution. Although the polyclonal RPRML antibody used in this study is listed as validated for immunohistochemical use by its manufacturer (https://www.abcam.com/rprml-antibody-ab204896.html), no information or scientific references could be found about this validation. In the current study, we validated RPRML antibody

showing that it is specific for Western Blot analysis of ectopically overexpressed RPRML protein. Moreover, positive immunostaining of RPRML correlated with transcript expression in gastric cancer cell lines, also suggesting specificity of RPRML antibody for IHC. Still, some antibodies may recognize proteins in their denaturated form but not in their native conformation (Bordeaux et al., 2010). Therefore, future research should take in to account that further validation is needed to strictly confirm the specificity of RPRML antibody for IHC applications. It is recommended that this validation consider the immunocytochemical assessment of overexpression and silencing experiments.

Based on the impact on survival of RPRML expression and its high homology to the RPRM putative tumor suppressor gene, we evaluated the effect of RPRML overexpression on biological characteristics associated with tumor progression *in vitro*. Our findings indicate that RPRML significantly inhibits clonogenic capacity and anchorage independent growth in the AGS cell line. These assays, considered the gold-standard to evaluate malignant transformation *in vitro*, strongly suggest that RPRML acts a tumor suppressor gene in gastric cancer.

Uncontrolled cell cycle progression and proliferation are key hallmarks of cancer (Hanahan & Weinberg, 2011). In normal cells, progression of the cell cycle is strictly regulated to ensure correct DNA replication, repair and cell division. Transition through each phase depends on the activation and heterodimerization of phase-specific cyclin subunits and cyclin-dependent kinases (Cdks) (Hochegger et al., 2008). Cyclins and Cdks are in turn regulated by a coordinated network of different activators and inhibitors in response to different DNA damage and stress sensors. When damage is sensed, an arrest is induced causing a delay in the cycle. If the arrest is irreversible or the damage can't be repaired, additional signaling mechanisms are triggered to induce cell apoptosis. In the current study, we found that RPRML overexpression arrested the cell cycle at G2/M phase, leading to reduced proliferation of AGS cells, without inducing caspase-dependent apoptosis. Taken together, these observations support a role in tumor suppression and suggest that RPRML acts in the cell cycle checkpoints signaling pathway.

Two major checkpoints regulate the cell cycle at G2/M. The first, marked by activation and nuclear translocation of Cyclin B1-Cdc2 complex, regulates entry to mitosis to ensure correct DNA replication and repair (Lockhead et al., 2020). The second, marked by the activation of APC/C (anaphase-promoting complex/cyclosome) and degradation of Cyclin B1, regulates mitotic spindle assembly allowing correct

segregation of sister chromatids (Zhang et al., 2016). More research evaluating the status of cyclin B1, cdc2 and APC/C upon overexpression of RPRML will allow to clarify its role in the cell cycle.

Early studies of RPRM, the founding member of the Reprimo gene family, showed that its overexpression leads to a phase G2 arrest of the cell cycle, through inhibition of cdc2 activation and of the nuclear translocation of cyclin B1 (Ohki et al., 2000). One important aspect about gene families is that they often share high sequence identity which may confer structural and functional similarities (Demuth & Hahn, 2009; Todd et al., 2001). According to (Joshi & Xu, 2007), proteins with aminoacidic sequence identity above 50%, have more than 80% chance of functional conservation within the same Gene Ontology (GO) Biological Process. Thus, it is not rare that the member of the Reprimo family studied herein, RPRML, also participates in cell cycle regulation. However, gene duplication is considered a key evolutionary force for functional diversification (Magadum et al., 2013). Some well-known gene families such as the p53 family are evidence of this diversification, showing specialized sub-functionalization or circumstantial expression (Acharya & Ghosh, 2016; Tutar, 2012). Therefore, the differences in the N-terminal sequence between RPRM and RPRML suggest that they are likely to have diversified biological functions within the cell cycle signaling pathway.

Our findings show that stable transfection of RPRML does not induce caspase- dependent apoptosis. This result suggests the presence of an overlapping mechanism able to overcome sustained repression by RPRML and restart the cycle. However, other types of cell death independent of caspase 3 cleavage may also occur (Niikura et al., 2007). On the other hand, as reported by (Stanic et al., 2019), RRPML has an opposite effect on apoptosis during the embryonic development of zebrafish. RPRML knockdown increases caspase-3 activity in the hemogenic endothelium, hindering the formation of hematopoietic precursor/stem cells. Although it is possible that RPRML has different functions during development and cancer biological processes, its role in apoptosis of human cells remains inconclusive. Evaluation of different cell death mechanisms through Anexin V flow cytometry may help to better understand this result. More broadly, microarray analysis of gene expression profiles from RPRML overexpression and silencing experiments could guide future research on the different biological processes in which it might be involved.

Nevertheless, the results from our *in vitro* experiments have to be seen in the light of the following limitations: First, we could not detect endogenous RPRML protein by Western Blot analysis in cell lines

with positive transcript expression, nor after re-expression by treatment with 5-Aza. These results could be explained by several factors. For instance, some proteins may be expressed only under specific conditions (stimulus) or may have very short turn-over rates, which makes them difficult to detect by Western Blot without a known induction method. Several examples of this can be found in the literature. In fact, many proteins required for cell cycle regulation are rapidly degraded to allow phase transition (Hochegger et al., 2008). Namely, the putative tumor suppressors OVCA1 and CLU, with half-lives of less than two hours, could be detected by Western Blot only after treatment with the proteasome inhibitor MG132 (Lin et al., 2018; Rizzi et al., 2009). This approach could be useful for future studies of RPRML protein.

On the other hand, to date there is no reported experimental evidence of a protein product for RPRML gene. Despite manual curation and reviewing by UniProtKB/Swiss-Prot database (https://www.uniprot.org/uniprot/Q8N4K4), RPRML protein remains an *in-silico* prediction with evidence only at the transcript level. In this study, we showed that RPRML gene has coding potential through Western Blot analysis of ectopically overexpressed RPRML open reading frame. Furthermore, Ribo-seq analysis strongly supports the existence of RPRML protein. Still, definite proof would need clear identification of the endogenous product either by immunological techniques, such as Western Blot or immunocytochemistry; or by structural methods like mass spectrometry, X-ray crystallography, or nuclear magnetic resonance (NMR) spectroscopy.

Nevertheless, despite the existence of RPRML protein, there is a possibility that the functionality of RPRML observed in this study is due to the acting as a noncoding RNA (ncRNA). In the past decades, it has become clear that ncRNA perform important cellular functions. Emerging roles of ncRNAs vary from regulation of transcription and chromatin architecture inside the nucleus, to control of mRNA stability, translation and post-translational modifications in the cytoplasm (Yao et al., 2019). In addition, the perception of non-coding and coding status has dramatically changed by the fact that some coding mRNAs can also function as ncRNAs (ie, bifunctional RNAs), while ncRNAs may encode functional small peptides as well (<100 aminoacids) (Li & Liu, 2019). Perhaps one of the most iconic bifunctional RNA is TP53, which, in addition to its well-known coding functions, can regulate its own protein stability by directly binding and inhibiting its negative regulator Mdm2 (M. M. Candeias et al., 2006; Marco M. Candeias et al., 2008). Thus, this line of evidence suggests that we cannot rule out the possibility that RPRML exerts its functionality neither as a ncRNA nor as a protein.

Another limitation of this study was the use of a single cell line to evaluate RPRML functionality. Validation in additional cell line models is required to confirm the tumor suppressor properties of RPRML. Of note, the AGS cell line has been one of the most widely used and better characterized cell lines in gastric cancer-related research. It is derived from a primary stomach adenocarcinoma tissue with no prior chemotherapy treatment, thus it is generally considered a good study model of gastric cancer (Barranco et al., 1983). Nevertheless, it is important to mention that overexpression of RPRML in another gastric cancer cell line, Kato-III, did not affect its proliferative or malignant capacity (data not shown). This could be due to the fact that, unlike the AGS cell line, Kato-III cells have a complete deletion of TP53 and therefore a misrepresented cell cycle control pathway. Similar results have been previously reported by (Shirin et al., 1999), showing that Kato-III was resistant to changes in the cell cycle and apoptosis after induction by *H. pylori* infection. Of note, as described by (Saavedra et al., 2015), the AGS cell line has undetectable transcript expression of the formerly studied RPRM gene, thus suggesting that the newly characterized family member, *RPRML*, has an independent role in this cell line. Future studies evaluating functional interactions between RPRM and *RPRML* may help to better understand the biological role of this gene family.

In addition, we explored the potential mechanism regulating RPRML expression in gastric cancer. Tumor suppressor genes are often inactivated or partially silenced by genetic alterations and/or epigenetic mechanisms such as DNA methylation, non-coding RNAs or histone modifications (Berger et al., 2011). Since genetic inactivation of RPRML is a rare finding in the TCGA database, we evaluated DNA methylation as it is one of the most common inactivation mechanisms in gastric cancer (Padmanabhan et al., 2017). The classic experiment to evaluate targets of DNA methylation was first described by (Jones & Taylor, 1980) and is based on the evaluation of expression profiles after treatment with the demethylating drug 5-Aza. This drug is a cytidine analogue that inhibits DNA methyltransferases (DNMTs) causing genome-wide demethylation and therefore re-expression of genes silenced through this mechanism (Wisnieski et al., 2020). In the current study, treatment with 5-Aza restored RPRML transcript expression in two stomach cell lines with prior undetectable mRNA (SNU-16 and Hs746T), suggesting that RPRML is regulated by DNA methylation.

However, it is plausible that 5-Aza-induced reactivation of RPRML occur indirectly through re-expression of a transcriptional activator. Therefore, analysis of local DNA methylation patterns is necessary to determine methylation-associated silencing. Bisulfite modification is the base to asses

methylation status of individual CpG sites, enabling conversion of unmethylated cytosines to uracil, while methylated cytosines remain intact (Clark et al., 1994). Subsequent direct sequencing of PCR products allows to obtain the average methylation status in a population of cells (Yamashita et al., 2018). Through this strategy, we confirmed that RPRML transcript expression is regulated by DNA methylation in SNU-16 and Hs746T cell lines. Moreover, methylation data from the CCLE (Ghandi et al., 2019) correlated with transcript expression of the Hs746T cell line, further supporting this finding.

Conversely, 5-Aza treatment did not restore RPRML transcription in the AGS cell line, despite increasing dosage of the drug. This result suggests that additional mechanisms may restrict RPRML expression, which is a common finding in other tumor suppressor genes (L. H. Wang et al., 2019). For example, CDH1, a well-known tumor suppressor in gastric cancer, is a hotspot for genetic alterations, however, DNA methylation, non-coding RNAs and transcriptional repressors have also been reported to participate in its bi-allelic inactivation (Khouzam et al., 2017). Of note, data from the CCLE indicate there is no evidence of mutational inactivation of RPRML in the AGS cell line (Ghandi et al., 2019). However, densely methylated loci are often associated with a repressive chromatin state due to the direct interaction of DNMTs with histone remodeling enzymes (Zahnow et al., 2016). Therefore, several studies have shown that reactivation of some hypermethylated genes require the combination of 5-aza with histone deacetylases inhibitors such as Trichostatine A (He et al., 2015; Sato et al., 2003). Nevertheless, analysis of the TCGA STAD dataset showed increased methylation in tumors compared to NTAM, and a significant correlation between transcript and methylation levels. Thus, taken together, these results suggest that DNA methylation plays an important role in the regulation of RPRML expression in gastric cancer.

Aberrant DNA methylation occurs early in the course of gastric carcinogenesis (Yamashita et al., 2018). Gene-specific DNA methylation has been long recognized as a promising biomarker for non-invasive cancer detection and clinical-decision making, offering the opportunity for early detection and improvement of gastric cancer survival rate (Nian et al., 2017; Yamashita et al., 2018). According to (Pepe et al., 2001) an optimal biomarker, should be expedite and minimally invasive in order to be applied in routine clinical settings. Regardless of its role in gene expression regulation, multiple studies have demonstrated that tumor-specific methylated DNA can be recovered from different biological fluids, including peripheral blood (Sapari et al., 2012). Particularly, plasma appears to be more enriched in circulating tumor DNA than serum, increasing the sensitivity of diagnostic tests (Lee et al., 2020).

To explore the potentiality of methylated RPRML DNA as a non-invasive biomarker, we evaluated whether it could be detected in plasma samples from gastric cancer patients. Current guidelines from the Early Detection Research Network (EDRN) indicate that the first phase in the development of novel biomarkers should start with preclinical studies and case-control design to identify potentially useful candidates (Pepe et al., 2001). Following these guidelines, we developed an MSP assay, which allows fast assessment of the methylation status of a specific sequence based on sodium bisulfite conversion and PCR amplification, using methylation specific primers (Konishi et al., 2015). In spite of the exploratory nature of this analysis, our results show that methylated RPRML can be detected in the peripheral blood of gastric cancer patients. Furthermore, the detection rate in plasma from these patients is significantly higher than in healthy donors. Therefore, methylated RPRML DNA in plasma could potentially serve as a non-invasive biomarker for early gastric cancer detection.

To assess the accuracy of our previous finding, we also explored a quantitative and more sensitive strategy termed MethyLight assay (Konishi et al., 2015). Using this assay, methylated RPRML DNA significantly discriminated gastric cancer from low-risk OLGA patients. Here, we report a sensitivity of 56.0% (95%CI:34.93-75-60) and a specificity of 87.50% (95%CI: 76.85-94.45). Nevertheless, minimal accepted values depend on the clinical setting, the phase in biomarker development, the disease prevalence, and the definition of the reference control group (Bossuyt et al., 2015).

Although sensitivity and specificity are the most commonly reported parameters to evaluate the performance of a biomarker, likelihood ratios (LR) may be more intuitive for clinical decision-making and useful for comparison between different tests since they are independent of the disease prevalence (Timsit et al., 2018). The LR+ represents how likely is that a patient has the disease, given a positive test (ie, sensitivity/(1-specificity)) (Glas et al., 2003). On the contrary, the LR- represent how likely is that a patient with a negative test doesn't have the disease (ie, (1-sensitivity)/specificity) (Glas et al., 2003). In the current study, the LR+ was 4.48 (95%CI 2.15-9.35) and the LR- was 0.50 (95%CI 0.32-0.79), indicating it is 4 times more likely that a patient with a positive result of circulating methylated RPRML DNA has gastric cancer while a patient with a negative result is half as likely to have the disease. Usually, an LR+ greater that 10 or an LR- below 0.1 are considered to provide strong evidence of the usefulness of the diagnostic test (Timsit et al., 2018). However, the use of a single biomarker is one of the main concerns for the development of non-invasive screening tests due to the heterogenous nature of the disease, so this result should be interpreted by the potential additive value to multi-biomarker approaches (Pepe et

al., 2001). Thus, RPRML may contribute to increase sensitivity of a multi-biomarker panel without adding considerable false positives to the test.

To compare the performance of several candidate biomarkers, the diagnostic OR is preferred. This approach describes the probability of a positive test in the disease relative to the probability of a positive test in the control group (ie, LR+/LR-) (Glas et al., 2003). A recent meta-analysis compared the clinical performance of all methylation-based blood biomarkers proposed for gastric cancer detection (Wen et al., 2017). Data form this metanalysis is summarized in Table V. The homologous of RPRML, RPRM was among the most accurate biomarkers proposed in this metanalysis. The obtained OR for RPRML of 8.91 (95%CI:3.02-26.31) places it in the middle of the most promising biomarkers for gastric cancer detection, performing better than MLH1 and p15, known to be frequently hypermethylated in gastric cancer (Cancer Genome Atlas Research Network, 2014; Qu et al., 2013). However, a wide 95% CI was observed in all candidates from this metanalysis, indicating low precision accuracy and inconsistence between aggregated studies. Therefore, analysis in larger set of cases and independent cohorts are crucial to determine the best candidates for gastric cancer detection.

Table V. Odds Ratios for methylation-based blood biomarkers in pooled case-control studies for gastric cancer detection adjusted by random effects. *

GENE	*ODDS RATIO*	*95%CI*	*NUMBER OF STUDIES*
RPRM	*111.1*	*36.67-336.59*	*3*
RASSF1A	*64.15*	*32.29-127.47*	*5*
RUNX3	*63.66*	*13.42-302.02*	*6*
CDH1	*18.19*	*7.38-44.8*	*8*
APC	*15.6*	*1.24-196.14*	*4*
P16	*14.21*	*4.18-48.23*	*12*
P15	*7.92*	*2.41-26.09*	*4*
DAPK	*7.82*	*0.92-66.26*	*3*
MLH1	*6.81*	*2.84-16.35*	*6*
GSTP1	*5.75*	*1.05-31.62*	*3*
MGMT	*3.16*	*1.47-6.81*	*3*

**Data from "Promoter methylation of tumor-related genes as a potential biomarker using blood samples for gastric cancer detection", by Wen, Zheng and Hu et al., 2017. Retrieved from Oncotarget, 2017; 9(44). doi: 10.18632/oncotarget.20782.*

Additional studies have explored the potential use of multi-biomarker panels for gastric cancer detection (Anderson et al., 2018; Gue Shin & Chol Kim, 2016; Saliminejad et al., 2020). A 3-marker panel composed of ELMO1, ZNF569, and C13orf18 methylated DNA was able to detected 86% of gastric cancer with 95% specificity (Anderson et al., 2018); the combined methylation of RPRM and RUNX3 had a sensitivity of 82% and specificity of 89% (Saliminejad et al., 2020); and the multi-biomarker panel including methylation of PYCARD, APAF1, MINT1, and BRCA1 achieved 97.6% sensitivity and 66.3% specificity (Gue Shin & Chol Kim, 2016). However, to date, there are no validated clinical trials that show an effective predictive or diagnostic value for gastric cancer in a large set of cases.

It should be noted that as a phase I exploratory study, a small case-control design was used, with a random selection of gastric cancer cases and controls. This design may have included bias in the results, since variables such as sex, age, body mass index, TNM stage, H. pylori and Epstein Barr virus infection, among others, could potentially influence the methylation status of the biomarker (Qu et al., 2013). In addition, we did not evaluate whether the detected methylated RPRML DNA was directly derived from tumors. Circulating DNA in plasma may be released from multiple sources within the body. In healthy individuals, typically 70-90% of this DNA is derived from white blood cells (Sun et al., 2015; Xia et al., 2017). In contrast, plasma from cancer patients is enriched by 20-40% of DNA from the organ affected (Sun et al., 2015). Hence, the opportunity to develop liquid biopsies that may serve for disease diagnosis and monitoring as well. Future research should assess clonal identity of methylated RPRML DNA in plasma to determine its robustness as a biomarker of gastric cancer.

In this dissertation, we have shown that the detection of RPRML methylation can discriminate gastric cancer from controls in two independent set of cases and two different methodologies, regardless of the tissue of origin. On the other hand, the selection of the control group for the MethyLight approach included cases with confirmed diagnosis of low risk OLGA (0-II), which is the intended-use of the biomarker. In addition, we have developed a sensitive and high throughput strategy to detect RPRML methylation (ie, MethyLight) which is crucial for an optimal diagnostic test. In contrast, all studies referred in the metanalysis performed by (Wen et al., 2017) used the MSP assay, which is more time consuming and is able to detect only a few CpG sites, thus have inferior precision to detect methylated DNA. Further validation in a larger set of cases and combination with other biomarkers will determine whether RPRML methylation is a candidate for non-invasive multi-biomarker panel test to detect gastric cancer.

VII. CONCLUDING REMARKS

This dissertation constitutes the first functional characterization of RPRML gene in humans, opening the way for further research and translational applications. This gene is downregulated in gastric cancer and its silencing is a risk factor for poor prognosis in advanced stages of disease. Moreover, RPRML exert tumor suppressor properties in vitro, probably through participating in the cell cycle checkpoints pathway. Thus, it may contribute to tumor development and progression.

DNA methylation plays an important role in the regulation of RPRML expression and its detection in plasma DNA propose this gene as a candidate biomarker for non-invasive detection of gastric cancer.

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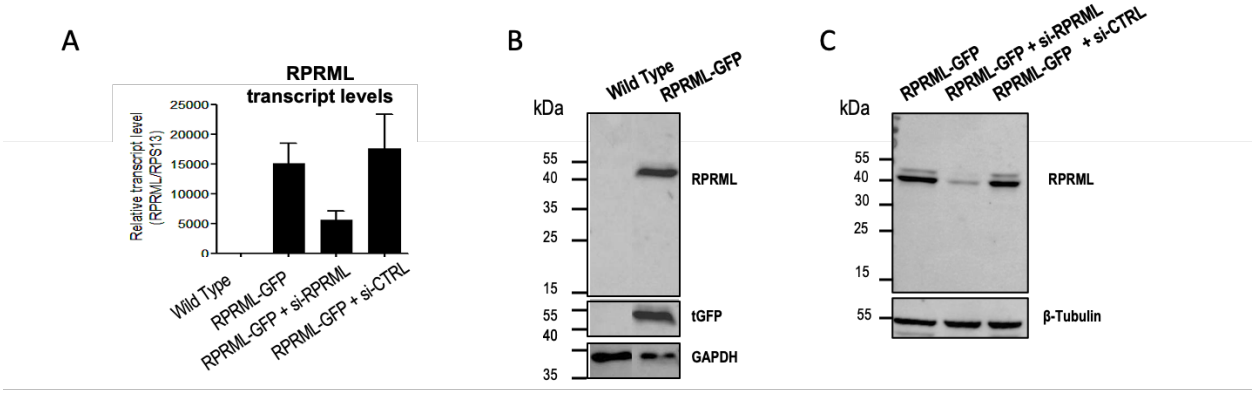
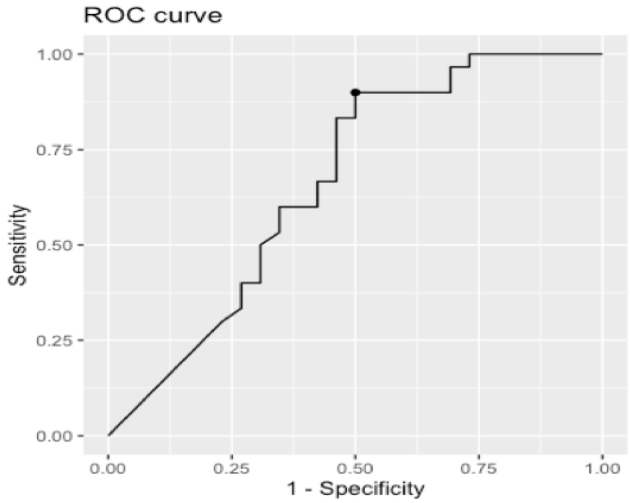
IX. SUPPLEMENTARY FIGURES

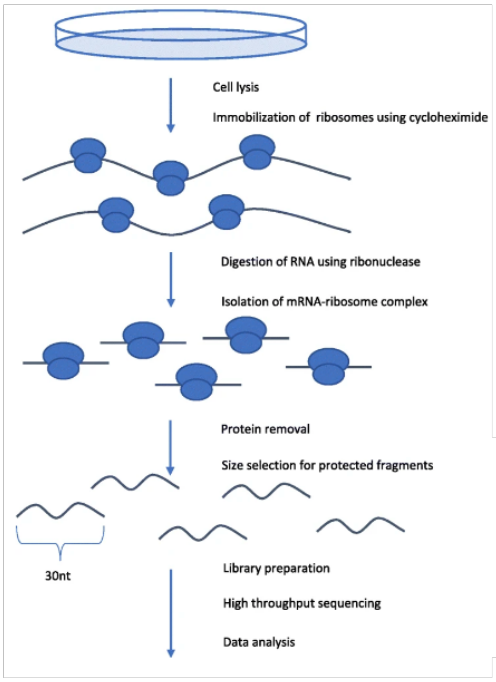


Supplementary Figure 1. RPRML antibody validation by Western Blot analysis.
A. Relative transcript expression of RPRML in AGS cells wild type, or transfected with RPRML-GFP. **B.** Western Blot analysis AGS wilt type or transfected with RPRML-GFP using anti-RPRML or anti turbo-GFP antibodies. **C.** Relative transcript expression of RPRML in AGS cells transfected with RPRML-GFP and treated with a pool of siRNA specific for RPRML (sc-94132, Santa Cruz Biotech) or control siRNA (sc-37007, Santa Cruz Biotech). **D.** Western blot analysis of C.



Supplementary Figure 2. Receiver Operating Characteristic (ROC) analysis of RPRML IHC Score for predicting an overall survival above the median survival time among advanced gastric cancer patients. Area under the curve was (AUC) was 0.665 and the maximum sensitivity and specificity were achieved with an IHC score of 0.162. With this cutoff point, sensitivity was 90% (95%CI: 73.47% to 97.89%), specificity 50% (29.93% to 70.07%), Positive predictive value (PPV) 67.5% (95%CI: 58.14% to 75.65%) and negative predictive value (NPV) 81.25% (95%CI: 58.08% to 93.13%).

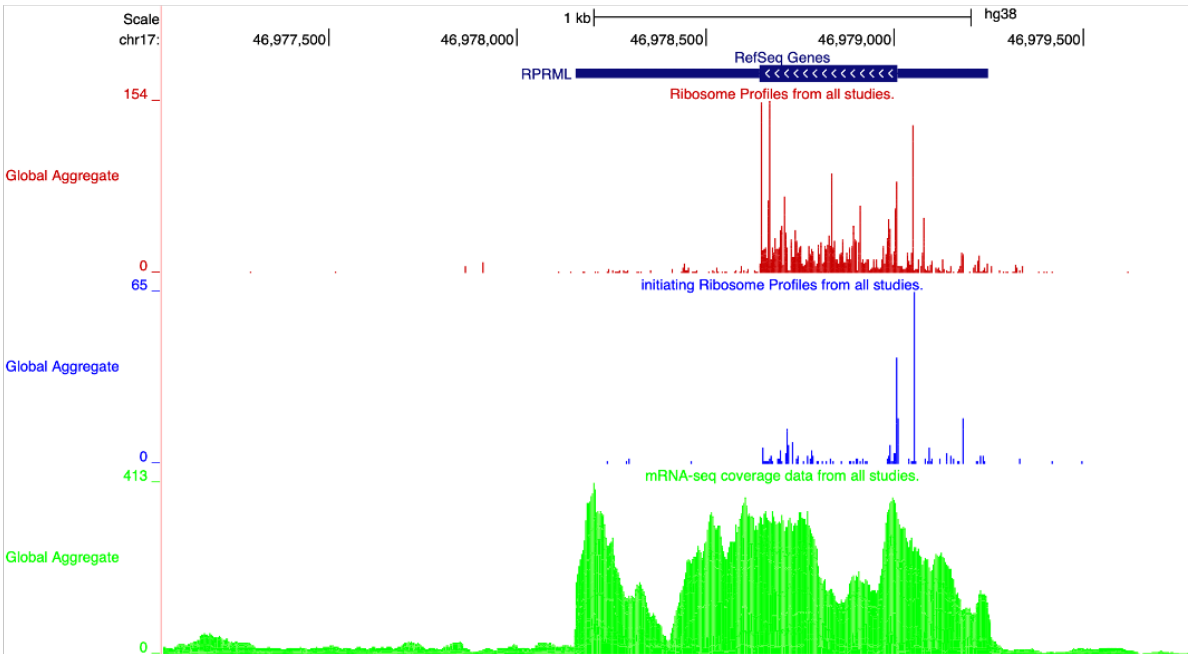
A

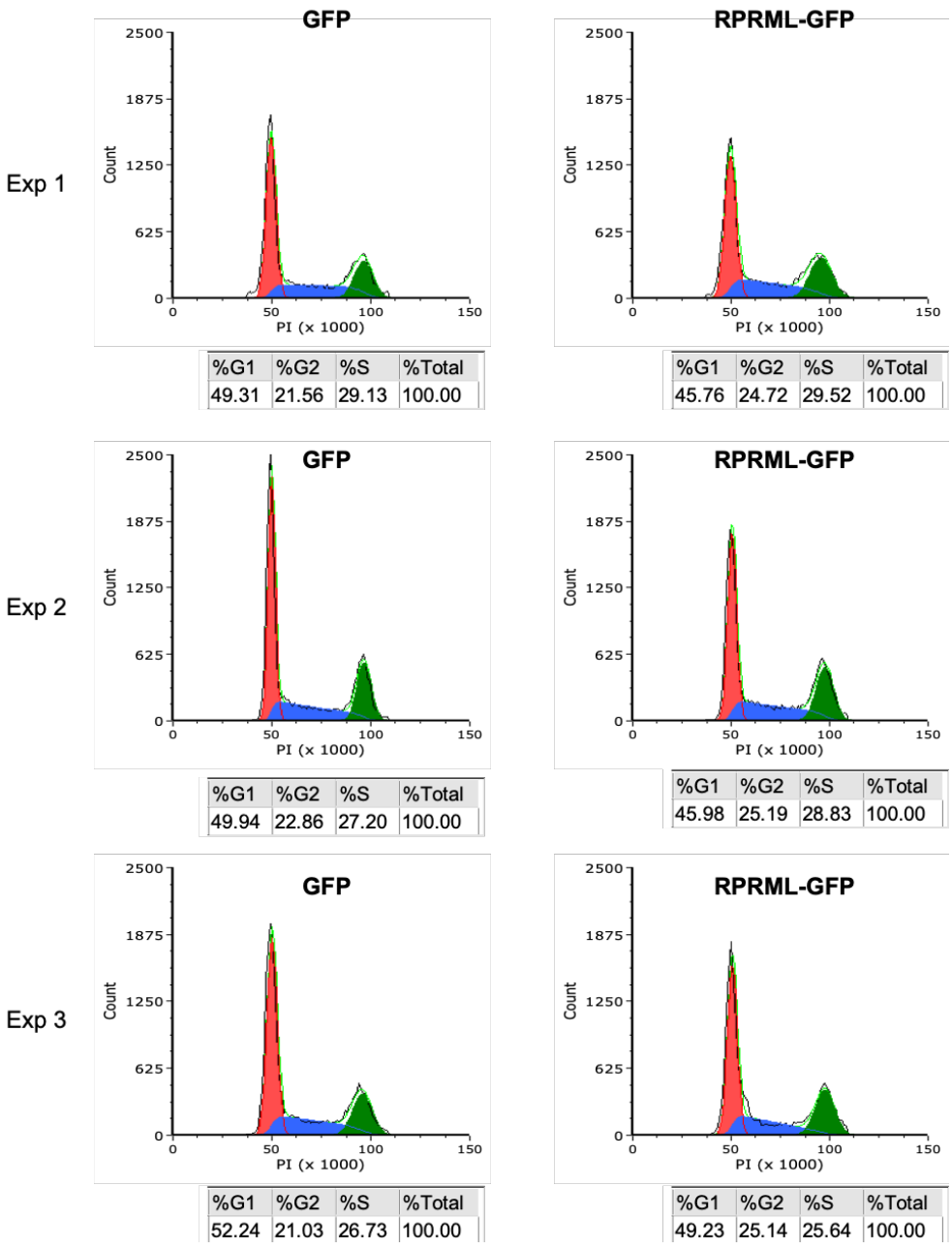


Supplementary Figure 3. Supporting evidence of the existence of RPRML protein product.

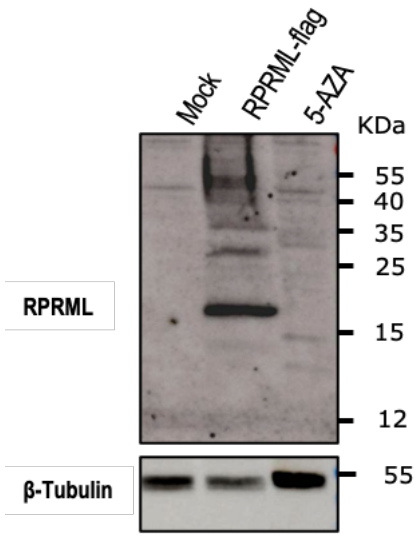
A. Schematic representation of Ribosome Profiling technique (Ribo-seq) extracted from Perkins, Mazzoni-Putman, Stepanova et al BMC Genomics, 2019 (https://doi.org/10.1186/s12864-019-5700-7). **B.** Ribosome profiling of RPRML gene. Plots were generated using GWIPS-viz web tool (http://gwips.ucc.ie) including aggregate Ribo-seq data from all available studies. On top, schematic representation of RPRML gene chromosomic location and structure. First panel (red) represents RNA read counts from elongating ribosomes, second panel (blue) represents RNA read counts from initiating ribosomes, and third panel (green) represents read counts of total mRNA-seq. The location of elongating and initiating ribosome read counts are consistent with the annotated coding region of the RPRML gene. Initiating ribosome profile show two peaks suggesting the existence of two start codons. One matching the current annotated start position (weaker peak) and a stronger peak located

B

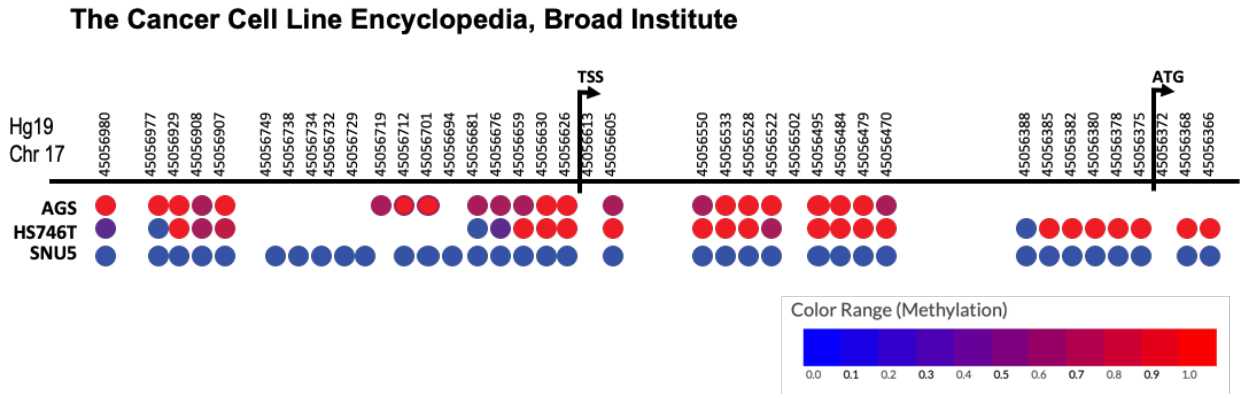




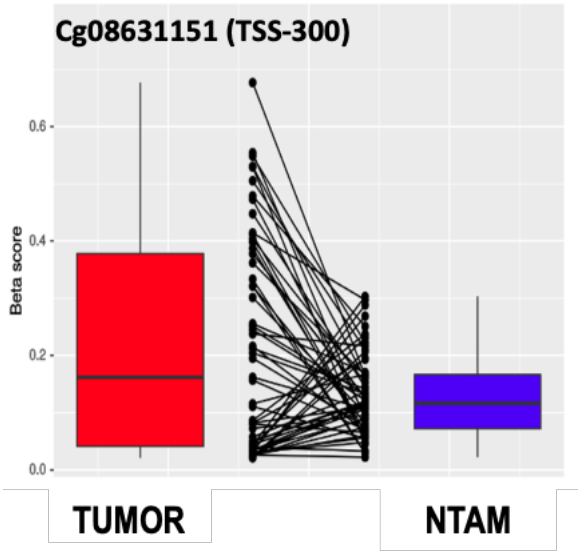
Supplementary Figure 4. DNA histograms of PI stained cells for cell cycle analysis of GFP and RPRML-GFP expressing AGS cells. Results from three independent experiments (Exp 1, 2, and 3). Data of 20.000 events/condition was recorded and frequency histograms were analyzed using FCS Express DeNovo Software using 1-cycle fitting and model 6. The percentage of cells at each phase is indicated in the tables below the DNA histograms.



Supplementary Figure 5. Western Blot analysis of RPRML in SNU-16 cell line mock (left lane) or treated with 1 uM of 5-Aza (right line). Protein lysate from AGS cells transfected with pCMV6-RPRML-DDK flag was used as a positive control (middle lane).



Supplementary Figure 6. CpG methylation status of RPRML DNA region surrounding the transcription start site (TSS). Schematic representation of RPRML DNA region and individual CpG location in chromosome 17 (Chr 17) from the Hg19 human genome assembly. TSS and ORF (ATG) are denoted by arrows. Beta values are represented by colors, ranging from blue for Beta values of 0 (unmethylated) to red for Beta value of 1 (fully methylated). Data downloaded from the Broad Institute Cancer Cell Line Encyclopedia.



Supplementary Figure 7. Differential Methylation in 59 paired stomach adenocarcinomas and NTAM samples from The Cancer Genome Atlas dataset (Illumina 27k methylation array). Beta values for probe Cg08631151 mapping to -300bp relative to RPRML transcription start site. Statistical Analysis: Wilcoxon signed rank test (p = 0.05)

X. SUPPLEMENTARY TABLES

Supplementary Table I. ROC curve analysis of RPRML IHC Score to discriminate between different clinicopathologic variables.

IHC Scoring	Parameter	AUC
1) Intensity	*OS> median survival**	0.454
	Positive Lymph node Metastasis	ND
	Positive Distant Metastasis	0.5195
2) Proportion of Stained cells	*OS> median survival**	0.4226
	Positive Lymph node Metastasis	0.4125
	Positive Distant Metastasis	0.5121
3) IHC Scor e= Intensity x Proportion of Stained Cells	*OS> median survival**	0.5627
	Positive Lymph node Metastasis	0.5727
	Positive Distant Metastasis	0.5316

**Median Overall Survival was 30 months*
ND: No discrimination

Supplementary Table II. Primers used for RT-PCR, Bisulfite Sequencing, MSP assay, and MethyLight assay

Assay	Sequence (5'->3')	Tm (°C)	%GC	Ta (°C)	Product Size
RT-PCR	Fw:ATGAACGCGACCTTCCTGAAC	65	52.4	60	90 bp
	Rv: GTGGGTGCGGTTTCCCA	65	64.7		
Bisulfite Sequencing RPRML	Fw: GGTGTTTAGGGGTAGG	46	56.3	55	219 bp
	Rv: TCCACCTCCTCCAAAC	49	56.3		
MSP/MethLlight RPRML	Fw: TTCGGTTTTAGTTTTTGCGTC	59.3	40.0	60	142 bp
	Rv: AACCGACTCCTACGATACGAA	55.1	47.6		
	Probe: CGGTTCGAGAGCGCGTAGGTAGTTA				
MSP/ MethyLight MYOD1	Fw:GGTTTTTTTAGGGAGTAAGTTTGTTAGG	54.5	35.7	57	105 bp
	Rv: CCAACTCCAAATCCCCTCTCTAT	56	47.8		
	Probe: TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC				

Tm: Melting Temperature, %GC: percentage GC content, Ta: Annealing temperature, bp: base pairs

Supplementary Table III. Overall number of patients at risk of death at each time point of the follow-up period according to the expression levels of RPRML among 56 advanced stage- gastric cancers (Reference form Figure 2).

Time	0 months	20 months	40 months	60 months	80 months	100 months	Total deaths
RPRML High	16	14	10	5	2	1	
RPRML Low	40	16	8	4	3	0	
Total number at risk	**56**	30	18	9	5	**1***	
Number of deaths		(56-30) = *26*	(30-18) = *12*	(18-9) = *9*	(9 -5) = *4*	(5 -1) = *4*	***55***

**Please note that at the end of follow-up (100 months) the total of patients alive is one, therefore the total number of deaths at 100 months is 55 from the initial 56 patients at risk.*