

ADRM1 gene amplification is a candidate driver for metastatic gastric cancers

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Abstract We searched for candidate target genes in metastatic gastric cancer, using comparative genomic hybridization (CGH) and mRNA expression array analysis of endoscopic biopsy samples collected from 32 patients. Recurrent amplicons included 17q21.2 (36,569,293–37,307,055), 8q24.13-q24.21 (126,357,475–130,159,285), and 20q13.33 (60,211,249–61,382,787). In this paper, we focused on the 1.1-Mb genomic region containing 24 genes in chromosome 20q13.33 (from 60,211,249 to 61,382,787), the third most frequent amplicon that was amplified in three of 32 patients (9.4 %), with \log_2 tumor/reference ratios ranging from 0.6 to 1.5. Of three genes in the 20q13.33 amplicon, *ADRM1* was chosen for functional analyses. *ADRM1* knockdown suppressed the proliferation of two human gastric cancer cells, SNU-601 and SNU-216. Overexpression of *Adrm1* promoted cell proliferation of conditionally-immortalized, mouse ImSt gastric epithelial cells, with increased S1 phase fraction and decreased expression of p21(Cip1). These results collectively indicate that *ADRM1* promoted gastric epithelial cell proliferation by cell cycle progression. Therefore, *ADRM1* is a candidate target gene in the chromosome 20q13.33 amplicon that may possibly be linked to development of gastric cancer.

Keywords *ADRM1* · Gastric · Cancer · Metastatic · Amplification

Abbreviation

CGH	Comparative genomic hybridization
EGFP	Enhanced green fluorescent protein
ANOVA	Analysis of variance
FISH	Fluorescence in situ hybridization
FFPE	Formalin-fixed: paraffin-embedded
FITC	Fluorescein isothiocyanate
FACS	Fluorescence-activated cell sorting
TMA	Tissue microarray

Introduction

Gastric cancer is the second most common cause of cancer deaths worldwide [1]. Cytotoxic chemotherapy is the mainstay treatment for metastatic gastric cancers, but the prognosis is generally poor. In addition to cytotoxic chemotherapy, HER2- and VEGFR2-targeted agents are currently available therapies shown to improve the prognosis of metastatic gastric cancers [2]. Given limited treatment options, there is an unmet need for novel targeted therapies to improve the prognosis of metastatic gastric cancer patients. Since gene amplifications are more relevant therapeutic targets for gastric cancers than single nucleotide variations or fusion genes [2], it will be a highly valuable study to identify novel target genes in recurrent amplicons in human gastric cancer. We previously reported the results of our expression array-based studies to identify biomarkers for chemotherapy resistance [3, 4]. In this parallel study, we sought to identify candidate target genes in metastatic

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gastric cancer patients, using array comparative genomic hybridization (CGH) analysis of the same endoscopic biopsy samples, and identified several recurrent copy number aberrations. Agilent array CGH technology, which was used in our previous studies [3], was used to evaluate copy number status of gastric cancer biopsy samples.

In this integrative CGH and mRNA microarray study, we evaluated copy number aberrations identified in 32 metastatic gastric cancer patients whose mRNA expression microarray data are also available [3]. We found that chromosome 17q21.2, 8q24.13-q24.21, and 20q13.33 were most recurrent amplification loci in our gastric cancer samples. Chromosome 20q13 is frequently amplified in gastric cancer [5, 6] and linked to high proliferative potential of tumors [7]. In contrast to 17q or 8q amplicon, 20q13 amplicon has not been fully defined for target genes [7]. Therefore, we focused on the 1.1-Mb amplicon in chromosome 20q13.33, which was found to be the third most frequent amplicon in our study patients. Among the 24 genes in the amplicon, *ADRM1/RPN13* demonstrated strong mRNA overexpression in gastric cancers and became the focus of this study. *ADRM1* is a 19S proteasomal cap-associated protein that interacts with a deubiquitinating enzyme UCH37 [8]. *ADRM1* has been proposed as a candidate driver gene in the 20q13.33 amplicon in ovarian and colorectal cancers [9, 10]. As a result of functional experiments, here we demonstrate that *ADRM1* amplification may contribute to gastric carcinogenesis by cell cycle progression, providing evidences that *ADRM1* is a candidate target gene in gastric cancer.

Materials and methods

Tissue samples were collected from metastatic gastric cancer patients by endoscopy before chemotherapy (cisplatin in combination with either fluorouracil or capecitabine) and frozen in liquid nitrogen from 2001 to 2006, under a protocol approved by the Institutional Review Board (IRB) of the National Cancer Center Hospital in Goyang, Korea. All patients signed IRB-approved informed consent forms. Tissue samples were collected and processed for total RNA and genomic DNA extraction as previously described [3]. Macrodissection was performed to ensure $\geq 50\%$ tumor content in all of the RNA and DNA samples. In parallel, grossly-normal gastric mucosa tissue samples were collected from the antrum of 21 healthy volunteers. Expression and CGH array analyses were performed using Affymetrix HG-U133A (Santa Clara, CA) and Agilent 4x44k HD-CGH (Agilent Technologies, Santa Clara, CA) arrays, respectively. For array CGH, human genomic DNA from multiple anonymous female donors (Promega, Madison, WI) was used as the reference DNA

[3]. Mean \log_2 tumor/reference ratio >0.5 for 5 or more consecutive probes was identified as the copy number gain.

For proliferation assays, 3×10^5 shEGFP-transduced and shADRM1-transduced SNU-601 cells were grown for 4 days in RPMI media with 10 % fetal bovine serum (FBS) on 150 mm dishes, and cells were counted daily using Countess (Invitrogen, Carlsbad, CA). Also, 1×10^4 empty vector-transduced and *Adrm1*-overexpressing, immortalized mouse gastric epithelial ImSt cells were grown for 4 days in RPMI media with 10 % FBS on 12 well plates. MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays were performed each day. Cell numbers and optical densities were compared using repeated measure ANOVA tests. Cell cycle analyses were performed using FACS. Fluorescence in situ hybridization (FISH) analyses were performed on formalin-fixed, paraffin-embedded (FFPE) slides for *ADRM1*-amplified gastric cancers using Cy3-labeled probes for *ADRM1* and FITC-labeled probes for 20p11.21. *ADRM1* immunohistochemistry (IHC) analyses were performed on *ADRM1*-amplified gastric cancers and also on a commercially available tissue microarray (TMA) slide containing 54 gastric cancers. Detailed experimental methods were described in *Supplementary Information*.

Results

ADRM1 gene amplification in gastric cancer tissue samples

Thirty two patients with metastatic gastric cancer were studied (Table 1). Median age was 62 years (range 27–74). There were 26 (81 %) males, and 13 Lauren intestinal

Table 1 Patient characteristics

	Gastric cancer (n = 32)	Healthy volunteer (n = 21)
Age (years)		
Median	62	53
Range	27–74	40–66
Male (%)	26 (81%)	15 (71.4 %)
Histology		
Intestinal	13 (41 %)	
Diffuse	19 (59 %)	
Stage, AJCC		
IV	32 (100 %)	
Median TTP (Mo)	5.3 (95 % CI 2.7–9.8)	
Median OS (Mo)	9.1 (95 % CI 6.8–15.4)	

AJCC American Joint Committee on Cancer, TTP time to progression, OS overall survival, Mo month

Table 2 Recurrent copy number gains and the *ADRM1* copy number status in study patients

Chr	Cytoband	Start	Stop	Log ratio ^a	No. patients
chr17	q21.2	36,569,293	37,307,055	2.03	16 % (5/32)
chr8	q24.13-q24.21	126,357,475	130,159,285	0.78	13 % (4/32)
chr20	q13.11-q13.2	41,522,907	52,785,621	0.64	9 % (3/32)
chr10	q26.13	123,264,524	123,458,608	4.61	9 % (3/32)
chr20	q13.33	60,211,249	61,382,787	0.71	9 % (3/32)

ADRM1 copy number status

Sex/age/histology	Cytoband (chr20)	Start	Stop	Log ratio ¹	<i>P</i>
M/70/diffuse	q13.33	60,211,249	62,343,424	1.53	3x10 ⁻⁴²
F/63/diffuse	q11.33-q13.33	32,934,124	62,343,424	0.62	1x10 ⁻¹⁰⁹
M/63/intestinal	q13.31-q13.33	54,958,391	61,382,787	0.71	2x10 ⁻²³

^a Median log₂ ratio of tumor/reference DNA (human genomic DNA from multiple anonymous female donors)

histologic types (41 %). Median percentage of tumor nuclei was 60 % (range 50–90).

Recurrent amplicons in our study patients included 17q21.2 (36,569,293–37,307,055 (n = 5)), 8q24.13-q24.21 (126,357,475–130,159,285 (n = 4)), and 20q13.33 (60,211,249–61,382,787 (n = 3)) (Tables 2 and S1). In contrast to 17q or 8q amplicon, 20q amplicon has not been fully defined for target genes, although it is frequently amplified in gastric cancer. Therefore, this study focused on the 1.1-Mb amplicon in chromosome 20q13.33 containing 24 genes (60,211,249–61,382,787), the third most frequent amplicon (Table 2). Log₂ tumor/reference ratio of the 20q13.33 amplicon ranged from 0.6 to 1.5. Two of the three patients with 20q13.33 amplification co-amplified 8q24.13-q24.21 (*MYC* locus).

It was postulated that target genes in the 20q13.33 amplicon may be overexpressed in mRNA and protein in corresponding tissue samples. To search for candidate target genes in the 20q13.33 amplicon, we compared the biopsy tissue mRNA expression microarray data between the three patients harboring 20q13.33 amplification and 21 healthy volunteers [4]. Three of 24 genes in the common amplicon in chromosome 20q13.33 (*ADRM1*, *TCFL5*, and *C20orf20*) were overexpressed by >twofold (*p* < 0.001) in three patients harboring 20q13.33 amplification, compared with healthy volunteers (Tables 3 and S2). We applied an additional filter to this gene list, using 2,446 genes differentially expressed between pre-chemotherapy samples and post-chemotherapy samples collected at the time of disease progression in chemotherapy responders (acquired resistance signature) [4]. Of three genes overexpressed in cancer patients, only *ADRM1* was included in the acquired resistance signature (1.3-fold overexpression in chemoresistant samples compared with pretreatment samples) [4]. Therefore, *ADRM1* amplification was subject to subsequent functional analyses.

Table 3 mRNA expression levels of genes in the common amplicon that were overexpressed in three patients with chromosome 20q13.32 amplification by >twofold at *p* < 0.001

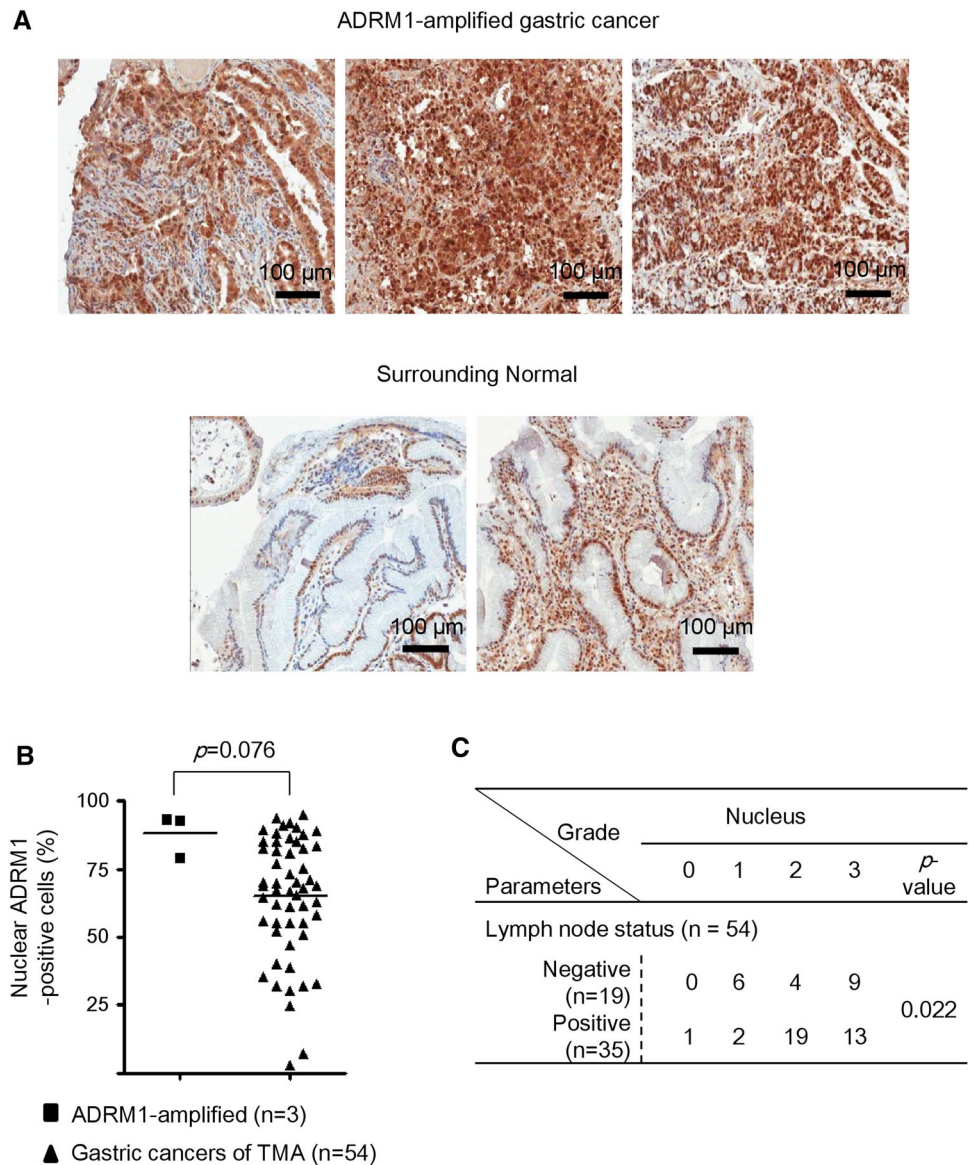
Gene name	Probe set	Ratio ^a	<i>p</i>
<i>ADRM1</i>	201281_at	2.7	<0.0001
<i>TCFL5</i>	204849_at	2.2	0.0001
<i>C20orf20</i>	218586_at	2.8	<0.0001

^a Affymetrix U133A signal ratio of cancer patients with 20q13.33 amplification (n = 3) to healthy volunteers (n = 21)

ADRM1 gene amplification was confirmed by FISH in all of the *ADRM1*-amplified patient tissue samples (Fig S1). When *ADRM1* immunohistochemistry was performed on FFPE slides for these tumors to confirm correlation between *ADRM1* amplification and protein overexpression, all of three *ADRM1*-amplified samples overexpressed nuclear *ADRM1* protein in cancer cells compared with surrounding histologically-normal gastric mucosal epithelial cells (Fig. 1a) and 54 gastric cancers of a TMA slide with unknown *ADRM1* copy number status (*p* = 0.076) (Fig. 1b). Interestingly, among 54 gastric cancers represented in TMA, there was trend of the higher *ADRM1* nuclear grade with positive lymph node involvement (Fig. 1c; *p* = 0.022).

According to mRNA expression array results from the whole study patients, *ADRM1* mRNA expression level (201281_at) was significantly higher in 32 gastric cancers than in 21 normal mucosal biopsy samples (*p* < 10⁻⁷; 2.3-fold). When the expression analysis was extended to a separate group of 92 metastatic gastric cancer patients whose samples were not subject to CGH array analysis [3], *ADRM1* was also overexpressed in 92 cancer patients than in 21 healthy volunteers (*p* < 10⁻⁷; 2.2-fold).

Fig. 1 Immunohistochemical analyses for *ADRM1* in tissue samples of three *ADRM1*-amplified cases. **a** *ADRM1* immunohistochemistry images of three *ADRM1*-amplified gastric cancers. Histologically-normal gastric mucosa surrounding two tumors is also shown. **b** Comparison of the percentage of nuclear *ADRM1* positivity between three *ADRM1*-amplified gastric cancers and gastric cancers represented in tissue microarray with unknown *ADRM1* copy number status. **c** *ADRM1* nuclear grade of gastric cancers represented in tissue microarray according to lymph node involvement. Chi square test was used to calculate the *p* value



ADRM1 promoted cell proliferation by cell cycle progression

Then, we moved on to evaluate functional impact of *ADRM1* in proliferation of gastric epithelial cells. *ADRM1* knockdown suppressed the monolayer growth of SNU-601 human gastric cancer cells (*p* for repeated measure ANOVA = 0.005) (Fig. 2a, b). *ADRM1* knockdown decreased the S phase fraction from 55 to 49 % in SNU-601 cells (*p* = 0.005) (Fig. 2c). Western blot analyses demonstrated the modest increase in protein level of p21(Cip1) (Fig. 2a). *ADRM1* knockdown suppressed the proliferation of another gastric cancer cell line, SNU-216 (*p* = 0.034; Fig S2).

ImSt is a conditionally immortalized stomach epithelial cell line isolated from the gastric epithelium of transgenic mice with a temperature-sensitive mutation of the simian virus 40 (SV40) large tumor antigen [11]. This cell line was

chosen for *Adrm1* overexpression experiments because of the premalignant phenotype (Fig. 2d). Overexpression of *Adrm1* enhanced the monolayer growth potential of ImSt cells (*p* for repeated measure ANOVA = 0.0002; Fig. 2e). S phase fraction of ImSt cells increased from 26 to 28 % after *Adrm1* overexpression (*p* = 0.0002; Fig. 2f). Protein expression level of p21(Cip1) was decreased after *Adrm1* overexpression (Fig. 2d), and increased after *Adrm1* knockdown (Fig S3). These results collectively indicate that *ADRM1* promotes gastric epithelial cell proliferation by cell cycle progression.

We recently established a primary cultured mouse gastric cancer cell line, NCC-S1, and the lung metastatic derivative cells, NCC-S1M, from a spontaneous gastric adenocarcinoma of a *Villin-Cre;Smad4^{F/F};Trp53^{F/F};Cdh1^{F/+}* mouse (Park JW et al., manuscript under review). *ADRM1* mRNA and protein expressions were higher in metastatic derivative

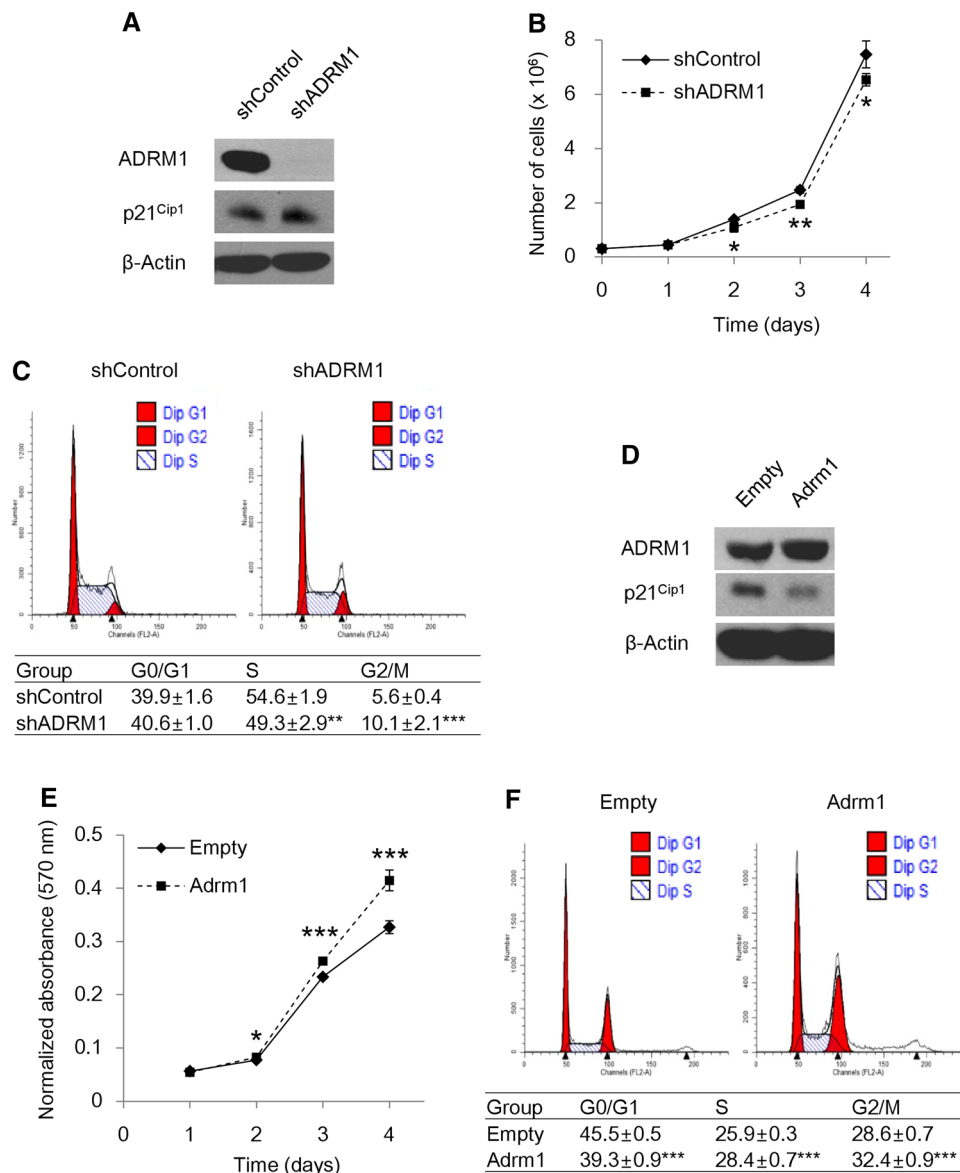


Fig. 2 Proliferation and FACS assays after ADRM1 modulation. **a** Western blot analyses. SNU-601 clones stably expressing lentiviral shADRM1 vector demonstrated undetectable expression level of ADRM1 and modest overexpression of p21(Cip1), as compared with control vector-transduced SNU-601 clones. **b** Growth curves of control-transduced and shADRM1-transduced SNU-601 cells. Error bars indicate SD from triplicate cell counting experiments. Student *t* test was used to compare cell number at each day (**p* < 0.05; ***p* < 0.01). **c** FACS cell cycle analyses on control-transduced and shADRM1-transduced SNU-601 cells on Day 4. Percentage (mean ± SD) of each phase is also shown (lower panel). Differences in S phase fraction were compared using Student *t* test (***p* < 0.01

and ****p* < 0.001). **d** Western blot analyses. ImSt clones stably overexpressing Adrm1 demonstrate decreased expression level of p21(Cip1), as compared with empty vector-transduced ImSt cells. **e** Growth curves of empty vector-transduced and Adrm1-overexpressing ImSt cells. Error bars indicate SD from quadruplicate MTT experiments. Student *t* test was used to compare cell number at each day (**p* < 0.05; ***p* < 0.001). **f** FACS cell cycle analyses on empty vector-transduced and Adrm1-overexpressing ImSt cells on Day 1. Percentage (mean ± SD) of each phase is also shown (lower panel). Differences in S phase fraction were compared using Student *t* test (***p* < 0.001)

NCC-S1M than in parental gastric cancer cell line NCC-S1 (Fig S4), with no alteration in *Adrm1* gene copy number status (A_53_P148343) according to array CGH analyses (data not shown).

Since ADRM1 is a substoichiometric subunit of the 26S proteasome, we planned to explore if some other proteasome

subunits are overexpressed in *ADRM1*-amplified cells. According to real-time PCR analyses on four selected gastric cancer cells, the copy number gain of *ADRM1* was documented in SNU-216 cells only (Fig. 3a). According to Western blot analyses, protein expression of ADRM1 in SNU-216 cells was higher than the other gastric cancer cell

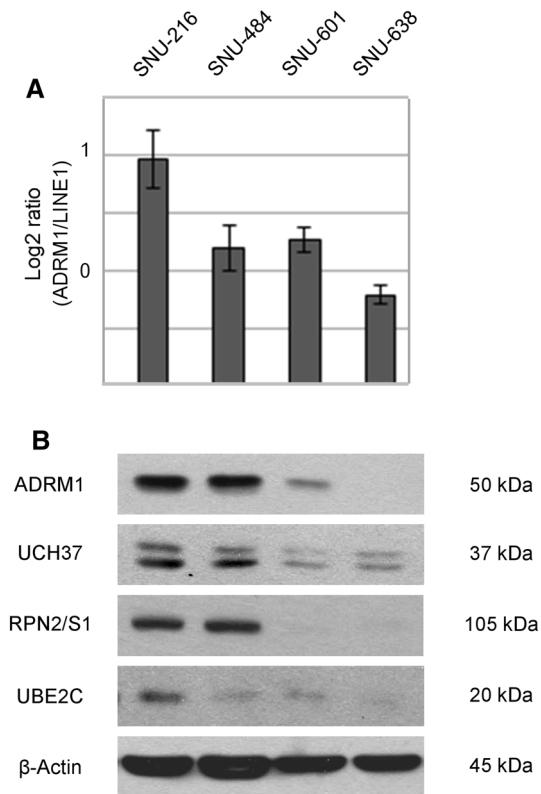


Fig. 3 ADRM1 copy number status and expression of related proteins in human gastric cancer cell lines. **a** *LINE1*-normalized genomic DNA copy number status of ADRM1 in four selected human gastric cancer cell lines, according to real-time PCR. Error bars indicate SD from triplicate experiments. **b** Western blot analyses on ADRM1 and selected proteins functionally related to ADRM1 in the same sets of cell lines

lines without *ADRM1* gene amplification (Fig. 3b). Compared with cell lines without *ADRM1* amplification, *ADRM1*-amplified SNU-216 cells also express higher protein levels of a proteasome 26S subunit RPN2/S1 [12], E2 ubiquitin-conjugating enzyme UBE2C [13], and ADRM1-interacting partner UCH37 [14–16] (Fig. 3b).

Discussion

Copy number aberrations have been exclusively studied using resected gastric cancer specimens so far, given the lack of available biopsy tissue samples from metastatic patients. In this regard, this is a unique study to evaluate the association of copy number aberration with gastric carcinogenesis. Despite relatively high frequency of chromosome 20q13.33 amplification in gastric cancer [5–7], target genes in this genomic region have not been clearly defined [7]. Chromosome 20q is frequently amplified in common solid tumors, and deletion of this arm is very rare [17–20]. According to a study performed by Tabach et al., 20q13

amplification is linked to high proliferative potential, and ADRM1, UBE2C, CSE1L, RPN2, C20orf45, MYBL2, TOMM34, AURKA, RAE1, PFDN4, PSMA7, RPS21 and VAPB are suggested as key cancer initiating genes, given overexpression in several cancers with advanced stage and poor prognosis [7].

Our study is the first to propose a possible role of *ADRM1* amplification in gastric cancer. ADRM1 has been proposed as a candidate driver gene in the 20q13.33 amplicon in ovarian and colorectal cancers [9, 10]. ADRM1 overexpression increases proliferation, migration, and growth in soft agar in ADRM1-amplified OAW42 ovarian cancer cell lines, and knockdown of ADRM1 results in apoptosis with dysregulation of CDK-activating kinase assembly factor MAT1 [9]. Knockdown of *Adrm1* by shRNA in human colon carcinoma RKO cells inhibits their anchorage-independent growth, cell migration as well as cell proliferation through inducing apoptosis and cell cycle arrest at the G1 phase [10].

ADRM1 is a 19S proteasomal cap-associated protein that interacts with UCH37, a deubiquitinating enzyme [8]. Our data suggests that *ADRM1* gene amplification may contribute to gastric carcinogenesis by cell cycle progression through enhanced degradation of cell cycle checkpoint proteins such as p21(Cip1). These results are consistent with a recent study performed by Anchoori et al., in which the pharmacologic inhibition of ADRM1 by RA190 reduces growth of multiple myeloma and ovarian cancer xenografts by stabilizing p53 and p53-regulated genes, such as p21, through proteasome inhibition [21].

In our 32 study patients, *ADRM1* was the third most frequent target for gene amplification, and the amplification was accompanied by ADRM1 overexpression. ADRM1 overexpression promoted monolayer growth of ImSt cells, while ADRM1 knockdown led to modest, but significant suppression of proliferation of gastric cancer cells. Therefore, *ADRM1* may be a candidate target gene for chromosome 20q13.33 gene amplification possibly linked to the development of gastric cancer.

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Conflict of interest Authors have nothing to disclose.

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