ATP11A is a novel predictive marker for metachronous metastasis of colorectal cancer

NORIKATSU MIYOSHI[1], HIDESHI ISHII[1,2], KOSHI MIMORI[2], FUMIAKI TANAKA[2], KENNICHI NAGAI[1], MAMORU UEMURA[1], MITSUGU SEKIMOTO[1], YUICHIRO DOKI[1] and MASAKI MORI[1]

[1]Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita, Yamadaoka 2-2, Osaka 565-0871; [2]Department of Molecular and Cellular Biology, Division of Molecular and Surgical Oncology, Kyushu University, Medical Institute of Bioregulation, Tsurumihara 4546, Beppu, Ohita 874-0838, Japan

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Abstract. The adenosine triphosphate-binding cassette transporter-homologous gene *ATP11A* belongs to an extended family of adenosine triphosphate-binding cassette transporters. We analysed the *ATP11A* gene in 7 colorectal cancer cell lines and 95 paired cases of colorectal cancer and non-cancerous regions to demonstrate the importance of *ATP11A* expression in colorectal cancer prognosis. *ATP11A* was expressed in the 7 colorectal cancer cell lines. *ATP11A* mRNA expression was higher in colorectal cancer tissue than in corresponding normal tissue (P<0.001). Patients with high *ATP11A* expression showed a poorer disease-free survival rate compared to those with low expression (P<0.001), thus indicating that increase in *ATP11A* expression was an independent predictor of metachronous metastasis of colorectal cancer. The present study suggests that *ATP11A* is a useful predictive marker of metastasis in colorectal cancer patients.

Introduction

Cancer is a major public health problem in developed countries. In Japan, the incidence of colorectal cancer (CRC) has significantly increased in recent years with changes in lifestyle (1). CRC is now one of the most important causes of death from neoplastic disease in Japan (1). The necessity of intensive follow-up and adjuvant therapy for CRC has been recently proposed in view of predicting recurrence and metastasis in curative surgical resection cases (2-4). Identification of genes responsible for development and progression of CRC and an understanding of their clinical significance are critical for diagnosis and adequate treatment of the disease.

Correspondence to: Dr Masaki Mori, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita, Yamadaoka 2-2, Osaka 565-0871, Japan
E-mail: mmori@gesurg.med.osaka-u.ac.jp

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The adenosine triphosphate-binding cassette (ABC) transporter-homologous gene *ATP11A* belongs to an extended family of ABC transporters that confer multi-drug resistance to cancer cells (5,6). In a previous study, *ATP11A* expression increased in leukaemia cells resistant to chemotherapy and the levels of *ATP11A* mRNA were 100-fold higher in cells that were intentionally made SCH66336-resistant (6).

We investigated the importance of the *ATP11A* gene by analysing it in 95 paired cases of CRC and non-cancerous regions as well as 7 CRC cell lines. We proposed the importance of *ATP11A* expression in prognosis evaluation, thus suggesting that *ATP11A* could be a novel predictive marker for prognosis of CRCs.

Materials and methods

Clinical tissue samples. Ninety-five patients (58 men and 37 women) with CRC underwent curative surgery at the Medical Institute of Bio-regulation at Kyusyu University from 1993 to 2001. We followed 61 of the 95 patients for over 5 years after the primary operation. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after receiving informed consent in accordance with the institutional guidelines. Each patient was definitively diagnosed with CRC based on clinico-pathological findings. Resected surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin and sectioned with haematoxylin and eosin as well as elastica van Gieson stains. The degree of histological differentiation, lymphatic invasion and venous invasion was examined. Immediately after resection, all specimens were frozen in liquid nitrogen and kept at -80˚C until RNA extractions were performed.

None of the patients received chemotherapy or radiotherapy prior to surgery. After surgery, the patients were followed up with blood examinations, including serum carcinoembryonic antigen and cancer antigen (CA19-9) level and imaging modalities such as abdominal ultrasonography and/or computed tomography and chest X-ray every 3-6 months. Clinico-pathological factors were assessed according to the tumour-node-metastasis (TNM) criteria of the International Union Against Cancer (7).

Cell lines and culture. Seven cell lines derived from human CRC (Caco2, DLD-1, HCT116, HT-29, KM12SM, LoVo and SW480) were obtained and maintained in Dulbecco's minimal essential medium containing 10% foetal bovine serum and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere. For the siRNA knockdown experiment, double-stranded RNA duplexes targeting human *ATP11A* (5'-AGGAGCGUCCAG UAGAUGAACACGC-3'/5'-GCGUGUUCAUCUACUGGA CGCUCCU-3', 5'-UUCAAAUGCUUAAACUUUGGGA UUG-3'/5'-CAAUCCCAAAGUUUAAGCAUUUGAA-3' and 5'-UGGAAAGGAAGAUGAAGUCGCAGGG-3'/ 5'-CCCUGCGACUUGAUCUUCCUUUCCA-3') were purchased (Stealth RNAi; Invitrogen, Carlsbad, CA, USA). Negative control siRNA (NC) was also purchased from Invitrogen. CRC cell lines were transfected with siRNA at a concentration of 20 μmol/l using lipofectamine (RNAiMAX; Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) for the time indicated and analysed by proliferation assay. All siRNA duplexes were used together as triple transfection. The growth rate of cultured cells was measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in 7 CRC cell lines to evaluate proliferation under *ATP11A* suppression. Each cell line with siRNA was compared to the wild-type and a negative control. Values were expressed as the mean ± standard deviation (SD) from independent experiments performed in triplicate.

RNA preparation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared using a modified acid guanidium-phenol-chloroform method with DNase (8). Reverse transcription was performed with 2.5 μg of total RNA as described previously (9) and a 143-bp *ATP11A* fragment was amplified. Two human *ATP11A* oligonucleotide primers for PCR were designed as follows: 5'-CACAGAGATACCCAGACAAC AGG-3' (forward) and 5'- CAACTGCACCAGAAATATGA TAAGG-3' (reverse). The forward primer was located in exon 2 and the reverse primer in exon 3. A PCR assay with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. cDNA from Human Reference total RNA (Clontech, Palo Alto, CA, USA) was used as a source for positive controls. Real-time monitoring of PCRs was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *ATP11A* and *GAPDH*. The amplification protocol consisted of 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec and elongation at 72˚C for 10 sec. The products were then subjected to a temperature gradient from 55˚C to 95˚C at 0.1˚C s^{-1}, with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumour and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Statistical analysis. Continuous variable data are expressed as the mean ± SD. The relationship between mRNA expression and clinico-pathological factors was analysed using the Chi-square test and Student's t-test. Kaplan-Meier survival curves were plotted and compared to the generalized log-rank test. Univariate and multivariate analyses to identify prognostic factors for overall survival were performed using the Cox proportional hazard regression model. All tests were analysed using JMP software (SAS Institute, Cary, NC, USA) and P-values <0.05 were considered statistically significant.

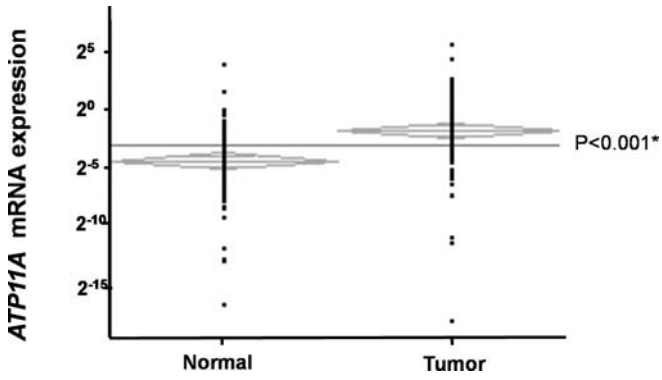

Figure 1. *ATP11A* mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase-polymerase chain reaction on 95 paired clinical samples showed that 80 of these cases (84.2%) exhibited higher levels of *ATP11A* mRNA in tumours than in paired normal tissues. The mean *ATP11A* mRNA expression level in tumour tissues (normalized by glyceraladehyde-3-phosphate dehydrogenase gene expression) was significantly higher compared to that of corresponding normal tissues (P<0.001; Student's t-test).

Results

ATP11A mRNA expression in clinical tissue specimens. We performed quantitative real-time RT-PCR analysis with paired primary and adjacent non-cancerous regions of CRCs. RT-PCR on 95 paired clinical samples showed that 80 of these cases (84.2%) exhibited higher levels of *ATP11A* mRNA in tumours than in paired normal tissues (Fig. 1). Mean *ATP11A* mRNA expression in tumour tissues was significantly higher than that for corresponding normal tissues (P<0.001; Student's t-test).

ATP11A expression and clinico-pathological characteristics. *ATP11A* expression was calculated by dividing the value of *ATP11A/GAPDH* expression of the tumour region by that of adjacent non-cancerous regions. For clinico-pathological evaluation, experimental samples were divided into 2 groups according to expression status. Patients with values more than the median *ATP11A* expression value (median, 5.800) were assigned to the high expression group and the others were assigned to the low expression group. Clinico-pathological factors related to *ATP11A* expression status of 95 patients are summarized in Table I and those of 61 patients that were followed over 5 years are summarized in Table II. Data indicate that *ATP11A* expression was correlated with tumour type, tumour invasion, lymph node metastasis, lymphatic invasion, venous invasion and metastasis.

Relationship between ATP11A expression and prognosis. Data show that the post-operative overall survival rate was

Table I. Clinico-pathological factors and *ATP11A* mRNA expression in 95 colorectal cancer patients.

Factors	High expression n=47 (%)	Low expression n=48 (%)	P-value
Age			
<68	26 (55.3%)	21 (43.8%)	0.259
≥68	21 (44.7%)	27 (56.2%)	
Gender			
Male	31 (66.0%)	27 (56.3%)	0.330
Female	16 (34.0%)	21 (43.7%)	
Histological grade			
Wel/Mod/	43 (91.5%)	47 (97.9%)	0.160
Por	4 (8.5%)	1 (2.1%)	
Tumour type			
Type 0-1	6 (12.8%)	13 (27.1%)	0.081
Type 2-4	41 (87.2%)	35 (72.9%)	
Tumour size			
<50 mm	23 (48.9%)	29 (60.4%)	0.260
≥50 mm	24 (51.1%)	19 (39.6%)	
Tumour invasion			
Tis	1 (2.1%)	4 (8.3%)	0.007
T1	3 (6.4%)	7 (14.6%)	
T2	3 (6.4%)	12 (25.0%)	
T3	33 (70.2%)	18 (37.5 %)	
T4	7 (14.9%)	7 (14.6%)	
Lymph node metastasis			
N0	21 (47.7%)	36 (75.0%)	0.002
N1-2	26 (55.3%)	12 (25.0%)	
Lymphatic invasion			
Absent	25 (53.2%)	38 (79.2%)	0.007
Present	22 (46.8%)	10 (20.8%)	
Venous invasion			
Absent	34 (72.3%)	45 (93.8%)	0.005
Present	13 (27.7%)	3 (6.2%)	
Metastasis			
M0	30 (63.8%)	46 (95.8%)	<0.001
M1	17 (36.2%)	2 (4.2%)	

Underlined values indicate statistical significance. Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma.

Table II. Clinico-pathological factors and *ATP11A* mRNA expression in 61 patients followed over 5 years.

Factors	High expression n=31 (%)	Low expression n=30 (%)	P-value
Age			
<68	17 (54.8%)	14 (46.7%)	0.523
≥68	14 (45.2%)	16 (53.3%)	
Gender			
Male	22 (71.0%)	16 (53.3%)	0.154
Female	9 (29.0%)	14 (46.7%)	
Histological grade			
Wel-Mod	30 (96.8%)	29 (96.7%)	0.981
Por	1 (3.2%)	1 (3.3%)	
Tumour type			
Type 0-1	2 (6.5%)	11 (36.7%)	0.004
Type 2-4	29 (93.5%)	19 (63.3%)	
Tumour size			
<50 mm	14 (45.2%)	18 (60.0%)	0.245
≥50 mm	17 (54.8%)	12 (40.0%)	
Tumour invasion			
Tis	0 (0%)	3 (10.0%)	0.001
T1	0 (0%)	5 (16.7%)	
T2	3 (9.7%)	8 (26.7%)	
T3	22 (71.0%)	10 (33.3 %)	
T4	6 (19.3%)	4 (13.3%)	
Lymph node metastasis			
N0	12 (38.7%)	24 (80.0%)	0.001
N1-2	19 (61.3%)	6 (20.0%)	
Lymphatic invasion			
Absent	16 (51.6%)	23 (76.7%)	0.041
Present	15 (48.4%)	7 (23.3%)	
Venous invasion			
Absent	21 (67.7%)	28 (93.3%)	0.011
Present	10 (32.3%)	2 (6.7%)	
Metachronous Metastasis			
Absent	15 (48.4%)	29 (96.7%)	<0.001
Present	16 (51.6%)	1 (3.3%)	

Underlined values indicate statistical significance. Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma.

lower in patients with elevated *ATP11A* expression (P=0.141). The median follow-up was 4.22 years (Fig. 2). We followed 61 of 95 patients over 5 years after the operation (median, 6.80 years). We also evaluated the metachronous metastasis-free over 5-year survival rate in these patients and found that the rate was significantly lower in patients with elevated *ATP11A* expression (P<0.001; Fig. 3). Table III shows univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that age (P=0.008), tumour type (P=0.042), tumour invasion (P=0.002), lymph node metastasis (P<0.001), lymphatic invasion (P<0.001), venous invasion (P=0.008) and *ATP11A* mRNA expression (P<0.001) were significantly correlated with post-operative metastasis. Multivariate regression analysis indicated that inclusion in the *ATP11A* mRNA high expression group [relative risk (RR), 8.79; 95% confidence interval (CI), 1.35-172.64; P=0.019] was an independent predictor of metastasis-free survival, as were age (RR, 5.57; 95% CI, 1.54-24.78; P=0.007) and venous invasion (RR, 4.56; 95% CI, 1.31-16.10; P=0.017).

Table III. Univariate and multivariate analyses for disease-free survival over 5 years (Cox proportional hazards regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (<68/≥68)	3.93	1.38-13.97	0.008	5.57	1.54-24.78	0.007
Gender (Male/Female)	1.56	0.57-4.91	0.389			
Histological grade (Por/Wel-Mod)	3.02	0.16-15.17	0.358			
Tumour type (Type 2-4/Type 0-1)	5.17	1.04-93.61	0.042	1.23	0.19-7.55	0.807
Tumour size (≥50/<50)	1.32	0.82-2.20	0.246			
Tumour invasion (T3-4/Tis-2)	9.31	1.89-168.23	0.002	1.06	0.12-4.97	0.948
Lymph node metastasis (N1-2/N0)	10.02	3.22-43.89	<0.001	3.22	0.94-15.54	0.063
Lymphatic invasion (Present/Absent)	5.51	2.04-17.35	<0.001	2.27	0.78-8.11	0.135
Venous invasion (Present/Absent)	4.05	1.46-10.63	0.008	4.56	1.31-16.10	0.017
ATP11A mRNA expression (≥5.8/<5.8)	20.07	4.08-362.59	<0.001	8.79	1.35-172.64	0.019

Underlined values indicate statistical significance. RR, relative risk; CI, confidence interval; Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma and mucinous carcinoma.

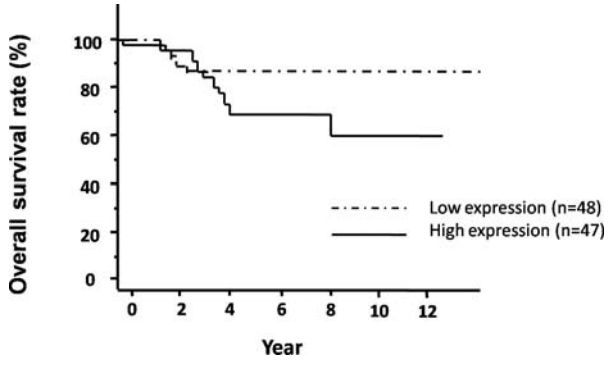

Figure 2. Overall survival curves based on *ATP11A* mRNA expression status of CRC patients. The post-operative overall survival rate was lower in patients in the high *ATP11A* expression group (P=0.141). The median follow-up was 4.22 years.

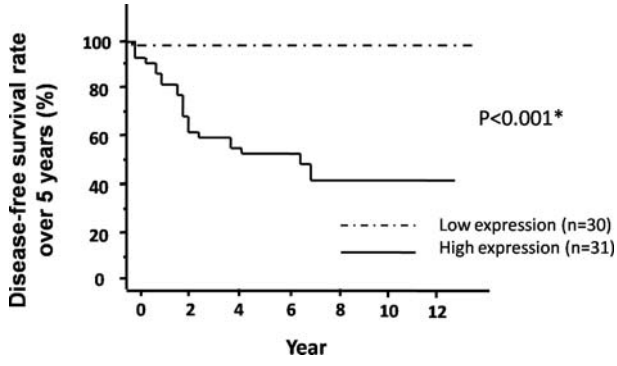

Figure 3. Disease-free survival curves based on *ATP11A* mRNA expression status of patients over 5 years after curative surgery for CRC. The post-operative disease-free survival rate was significantly lower in patients in the high *ATP11A* expression group (P<0.001). The median follow-up was 6.80 years.

In vitro assessment of ATP11A expression knockdown. Seven CRC cell lines were chosen for the proliferation study because their expression was higher than the median value of *ATP11A/GAPDH* in primary CRC specimens by RT-PCR. Significant reduction in *ATP11A* by siRNA was also confirmed by quantitative real-time RT-PCR in Caco2, HCT116, KM12SM and LoVo cell lines (NC and *ATP11A* siRNAs; P<0.05; Student's t-test; Fig. 4). Significant reduction in *ATP11A* expression was confirmed by seeding the cells ($1x10^5$) in 12-well dishes and culturing them for 96 h to determine proliferation. The results showed no significant difference in cell numbers between NC and *ATP11A* siRNA.

Discussion

ATP11A, also known as RP-11-120K24.1, ATPIH or ATPIS, is expressed in the heart, brain, muscle and liver of mice (10).

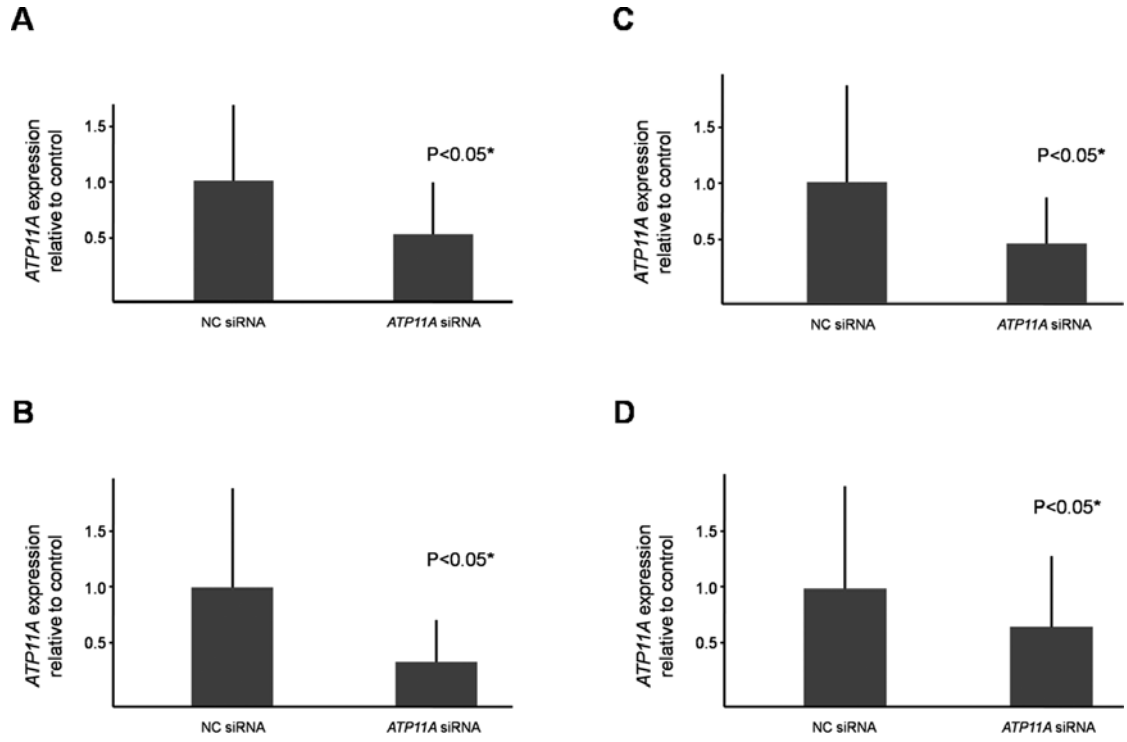

Figure 4. siRNA inhibition of *ATP11A* in 4 colorectal cancer cell lines. Suppression of *ATP11A* expression was confirmed by real-time reverse transcriptase-polymerase chain reaction. Reduction in the *ATP11A* siRNA experiment was significant compared to NC (P<0.05; Student's t-test) in 4 cell lines (A, Caco2; B, HCT116; C, KM12SM; D, LoVo). The results showed no significant difference in cell numbers between NC and *ATP11A* siRNA. NC, negative control.

It has also been reported to be expressed in human blood and colon (6).

The present study is the first to provide data indicating higher *ATP11A* expression in CRC and the possibility that *ATP11A* expression could be a useful predictive marker for metachronous metastasis of CRC.

Adjuvant chemotherapy for CRC after curative resection has been proposed; intensive follow-up is necessary (2-4). There are many recent studies related to less invasive surgery for CRC, such as laparoscopic and endoscopic surgery (11-14). For these cases, a predictive marker for tumour invasion as well as lymph node and distant metastasis would play a very important role in cancer diagnoses and treatments, especially as a novel marker independent of traditional TNM factors. Thus, the *ATP11A* expression profile may contribute to some kind of predictive diagnosis.

In a previous study, *ATP11A* was used as a drug transporter. Modulating *ATP11A* levels either through overexpression or siRNA knockdown caused decreased sensitivity of cells to SCH66336, thus indicating that *ATP11A* can directly transport SCH66336 out of cells (6).

The results of the present study showed that the metachronous metastasis-free survival rate was significantly lower in patients with elevated *ATP11A* expression; however, it was also shown that *ATP11A* expression was not related to tumour growth in CRC cell lines. Detailed investigation of *ATP11A* expression and adjuvant chemotherapy did not show any apparent correlation (data not shown). Furthermore, 33 of 61 (54.9%) cases in the present study had adjuvant chemotherapy, thus suggesting that *ATP11A* could be an independent predictive marker for metachronous metastasis in CRC patients; however, further investigation is necessary. Our results suggest a rationale for further study of *ATP11A* as a possible novel target for clinical cancer therapy.

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