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GASTRIC CANCER

Rationales for expression and altered expression of apoptotic protease activating factor-1 gene in gastric cancer

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Abstract

AIM: To elucidate the relationship between apoptotic protease activating factor-1 (Apaf-1) gene and gastric cancer.

METHODS: Thirty-five postoperative cancer and adjacent normal tissue samples were collected in the present study. Expression of the Apaf-1 gene in these samples was analyzed by semi-quantitative RT-PCR. Loss of heterozygosity (LOH) was used to determine whether there was loss of Apaf-1 gene in domain of 12q22-23 in the samples. Promoter methylation of Apaf-1 gene in the samples was analyzed by methylation specific (MSP) PCR.

RESULTS: The expression of Apaf-1 mRNA in gastric cancer tissue samples was 51%. The LOH frequency of D12S346, D12S1706, D12S327, D12S1657 and D12S393 was 33%, 8%, 58%, 12% and 42%, respectively. Fifty percent LOH was found at two sites and 17% LOH at three sites. Apaf-1 mRNA expression decreased significantly in 13 cases (r_s = 0.487, P = 0.003). The rate of Apaf-1 promoter methylation was 49% in gastric cancer tissue samples and 23% in para-cancerous tissue samples. Promoter methylation occurred significantly in 16 of 18 gastric cancer tissue samples with decreased expression of Apaf-1 mRNA r_s = 0.886, $P = 10^{-6}$).

CONCLUSION: The expression of Apaf-1 gene is low in gastric cancer tissues. Methylation of Apaf-1 gene promoter and LOH in domain of 12q22-23 are the main reasons for the expression and altered expression of Apaf-1 gene.

Key words: Gastric cancer; Apaf-1 gene; Loss of heterozygosity; Methylation

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INTRODUCTION

Formation and progression of gastric cancer are a continuous multiple-step process. Cell apoptosis is one of the main mechanisms for tumor genesis[1-3], and Apaf-1 protein is an important apoptosis factor which is expressed abnormally in a series of tumor studies[4-7]. It was recently reported that Apaf-1 gene is closely related to several cancer-inducing genes and tumor suppressor genes, such as p53 and Bcl2[8-10]. However, the expression condition of Apaf-1 gene in gastric cancer and its correlation with the genesis of gastric cancer remain unclear. This study was to study the relation between Apaf-1 gene expression and gastric cancer genesis in promoter domain.

MATERIALS AND METHODS

Materials

Thirty-five postoperative cancer tissue and adjacent normal tissue samples (> 5 cm away from the center of cancer) were collected from gastric cancer patients from January 2005 to September 2005 at No.1 and No.2 Affiliated Hospitals of China Medical University. All these patients were pathologically diagnosed according to the new TNM classification criteria of Union Internationale Contre le Cancer[11]. Of the 35 patients with progressive gastric cancer (25 males and 10 females with a mean age of 58.51 ± 12.24 years), 10 had moderately differentiated gastric cancer and 25 had poorly-differentiated gastric cancer, while 25 had lymphatic metastasis and 10 had no lymphatic metastasis. Tumor and adjacent normal tissues were isolated and maintained at -70℃. Total RNA extracting reagent Trizol (Invitrogen Co.), RT-PCR kit (TaKaRa Co.), methylating reagent (Sigma Co.) and Wizard DNA clean-up (Promega Co.) were used in the study.

Semi-quantitative RT-PCR analysis

Total RNA was extracted with Trizol reagent and cDNA

Table 1 Polymorphic sites, primers and amplification conditions (12q22-23)

Site	Repeat	Heterozygosity	Amplification length (kb)	Primer sequence	Reaction conditions
D12S327	Dinucleotide	0.8501	0.1820-0.2010	Aaa gtt tct gga tgg taa tat cg Aga gca aga cct tgt ctc aa	54℃ 40 s 72℃ 45 s
D12S1657	Dinucleotide	0.6439	0.1500-0.1600	Tcc taa aga tgg tgt gca t Aag ttc caa tgt tag tga acc	58℃ 40 s 72℃ 45 s
D12S393	Tetranucleotide	0.6410	0.2490	Att att gcc agg aca tta aac g Cct cac aca atg ttg taa ggg	58℃ 30 s 72℃ 40 s
D12S1706	Dinucleotide	0.8916	0.1190-0.1390	Cct atg att tcc cat caa gtt t Att att agg aga gcc ctg gg	57℃ 40 s 72℃ 40 s
D12S346	Dinucleotide	0.8400	0.1660	Tgc cc acct gcc tgt aac Aat gga ggg taa atg ccc g	58℃ 30 s 72℃ 40 s

was synthesized with reverse transcriptase and Oliga $(dT)_{20}$ primer. Apaf-1 primer sequence was designed with primer 3, including functional caspase recruitment domain (CARD), upstream primer (5'-TTGCTGCCCTTCTCC ATG AT-3'), downstream primer (5'-TCCCAACTGAAA CCCAATGC-3') and amplification length (334 bp). The internal reference primer was β-actin, including upstream primer (5'-GTGGGGCGCCCCAGGCACCA-3'), downstream primer (5'-CTCCTTAATGTCACGCAC GATTTC-3') and amplification length (498 bp). Both pairs of primers were added into 25 μL reaction system to react under the proper PCR reaction conditions (denatured at 94℃ for 45 s, renatured at 58℃ for 45 s and elongated at 72℃ for 60 s, for 35 cycles). RT-PCR products were isolated with 2% agarose gel, imaged through Alpha Image 2000 automatic imager and analyzed using Fluorchem V 2.0 Stand Alone software.

LOH analysis

Down-regulation of Apaf-1 gene expression was correlated with allele loss at its 5 sites (D12S327, D12S1657, D12S393, D12S1706 and D12S346). These 5 polymorphic sites were selected for LOH analysis. DNA was extracted from tumor and normal tissues using phenol/chlorine method. Primer sequences derived from genome database (GDB) and amplification conditions are listed in Table 1. Two percent agarose gel electrophoresis revealed target genes but no hybrid band in each specimen. PCR products were added into the denaturing buffer (98% formamide, 10 mmol/L EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol) at a ratio of 1:1, mixed uniformly, denatured at 97℃ for 10 min and immediately placed into ice. Fifteen μL of specimens was loaded in each hole, separated through 9% non-denaturing PAGE (in a 100 V low-temperature water bath for 2-3 h) and the electrophoresis results were analyzed through silver staining.

Methylation analysis at Apaf-1 promoter domain

DNA extracted from gastric cancer and adjacent normal tissues was treated with Na_2SO_3, purified and recovered with Wizard DNA clean-up (Promega Co.) for MSP analysis. A 49-378 base zone rich in CpG at Apaf-1 cDNA promoter domain was selected to design MSP primers, including upstream primer (5'-GAGGTGTCGTAG CGGTATTC-3'), downstream primer (5'-CGAAAATTA ACGAAATAAACGTC-3'), PCR reaction conditions (denatured at 94℃ for 45 s, renatured at 58℃ for 45 s and elongated at 72℃ for 60 s, for 38 cycles) and amplification length (221 bp). Methylating primers included upstream primer (5'-ATTTGAGGT GTTGTAGTGGTATTT G-3'), downstream primer (5'-ACCTCCAAAAATTAACAA AATAAACAT-3'), PCR reaction conditions (denatured at 94℃ for 45 s, renatured at 56℃ for 45 s, elongated at 72℃ for 60 s, for 38 cycles) and amplification length (221 bp).

Statistical analysis

Statistical analyses were performed by the SPSS13.0 software package (SPSS, Inc, Chicago, IL). Continuous Data were expressed as mean ± SD. Continuous variables were compared by *t*-test when appropriate, whereas categorical variables were compared by χ^2 test or Fisher's exact test when appropriate. Spearman rank correlation was analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Gene expression

The gene expression was increased 30%-70% in cancer tissue samples with an increased difference between Apaf-1/β-actin cDNA optical density ratios in gastric cancer and adjacent normal tissue samples. In the present study, Apaf-1expression decreased in 48.57% (17/35) of gastric cancer tissue samples (including expression loss in 2 cases), paired *t* test with SPSS13.0 statistical software showed that the relative Apaf-1 content was 0.96 ± 0.40 in adjacent normal tissue samples and 0.69 ± 0.36 in cancer tissue samples, respectively ($t = 3.518$, $P < 0.01$), and Apaf-1expression was significantly higher in adjacent normal tissue samples than in cancer tissue samples. Apaf-1 mRNA expression did not increase in tumor tissue samples (Figures 1 and 2).

LOH analysis

The data from 2 fragments (i.e. genome DNA was a certain marked heterozygote in normal tissue or adjacent normal tissue samples) were sufficient for LOH analysis, but the data from one fragment were not sufficient for LOH analysis. LOH meant that there was 1 band with less genome DNA PCR amplification product or 1

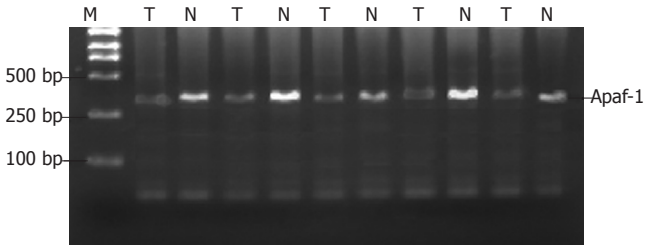

Figure 1 Expression of Apaf-1 mRNA. M: Marker, T: Tumor, N: Adjacent normal tissue, Apaf-1 mRNA: 334 bp, marker: DL2000.

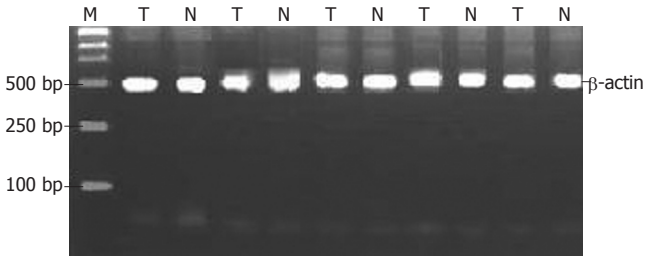

Figure 2 Expression of β-actin mRNA. M: Marker; T: Tumor; N: Normal adjacent tissue; β-actin mRNA: 498 bp; marker: DL2000.

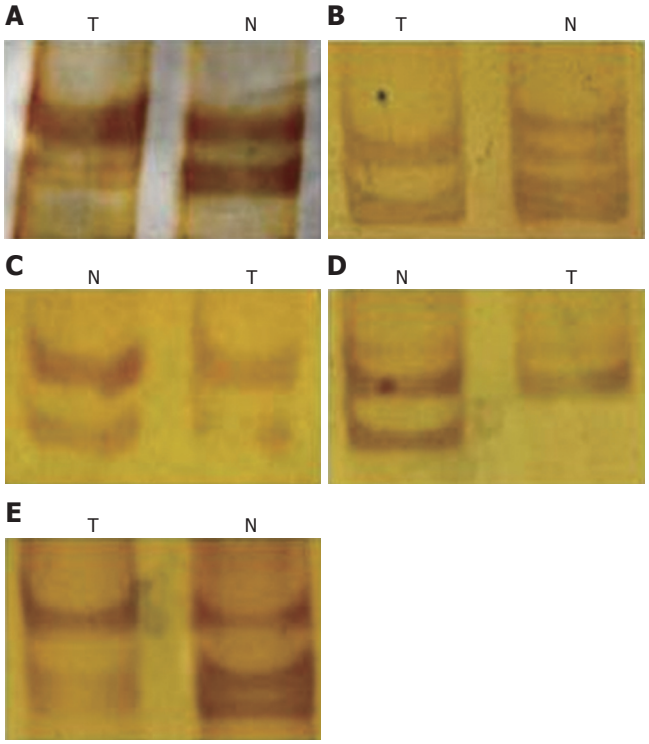

Figure 3 LOH analysis of polymorphic sites of D12S327 (**A**), D12S1657 (**B**), D12S393 (**C**), D12S1706 (**D**), and D12S346 (**E**). T: Tumor tissue; N: Adjacent normal tissue. Silver staining diagram showing Apaf-1 loss and LOH analysis showing allele loss at 5 polymorphic sites of Apaf-1 gene in gastric cancer tissue.

band with significantly weaker signal of up to 50% in cancer tissue samples than that in adjacent normal tissue samples. As shown by LOH analysis of 5 polymorphic sites for Apaf-1 gene in the present study, the LOH detection rate was 34.29% (12/35), 8.57% (3/35), 57.14% (20/35), 11.43% (4/35) and 42.86% (15/35) at single D12S346, D12S1706, D12S327, D12S1657 and

Table 2 Correlation of Apaf-1, LOH and Apaf-1 mRNA expression in gastric cancer tissue samples

		mRNA expression of Apaf-1 gene (*n*)		*r*s	*P*
		Down-regulation	Up-regulation		
More than 2 sites (*n*)	+	13	5	0.487	0.003
	-	4	13		

Table 3 Correlation of Apaf-1 methylation and Apaf-1 mRNA expression in gastric cancer tissue samples

		mRNA expression of Apaf-1 (*n*)		*r*s	*P*
		Down-regulation	Up-regulation		
Apaf-1 gene methylation (*n*)	+	16	1	0.886	10^{-6}
	-	1	17		

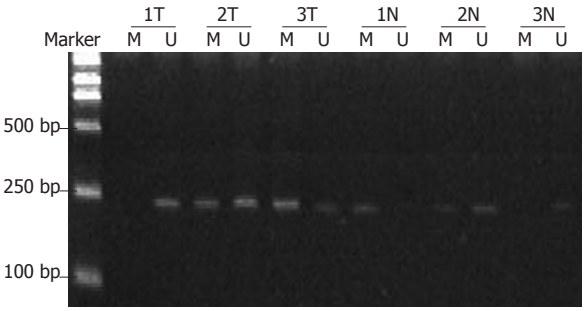

Figure 4 Methylation of Apaf-1 promoter. M: 212 bp methylation band; U: 221 bp non-methylation band; T: Gastric cancer tissue.

D12S393, respectively. However, the LOH detection rate was 51.43% (18/35) at more than 2 sites (16.67% at 3 sites) (Figure 3), suggesting that the LOH of Apaf-1 gene was correlated with the genesis of gastric cancer. Apaf-1 mRNA expression decreased significantly in 13 cases ($r_s = 0.487$, $P = 0.003$), indicating that the LOH of Apaf-1 gene was correlated with its decreased expression in gastric cancer (Table 2 and Figure 3).

Promoter methylation

MSP analysis of methylation conditions for Apaf-1 gene promoter showed that the methylation rate of Apaf-1 gene was 48.57% (17/35) in gastric cancer tissue samples and 17.14% (6/35) in adjacent normal tissue samples, respectively ($P < 0.05$), demonstrating that Apaf-1 methylation was correlated with the genesis of gastric cancer. Significant promoter methylation occurred in 16 of 18 gastric cancer tissue samples while the expression of Apaf-1 mRNA was decreased ($r_s = 0.886$, $P = 10^{-6}$), displaying that promoter methylation was correlated with decreased expression of Apaf-1 gene (Table 3 and Figure 4). Promoter methylation and LOH of Apaf-1 gene occurred at polymorphic sites in 17 of 18 gastric cancer tissue samples and the expression of Apaf-1 mRNA was decreased.

DISCUSSION

Human Apaf-1 gene is located at chromosome 12q23 to

encode cytoplasm protein (130 KD). As an important apoptosis factor, Apaf-1 gene participates in the pathway of mitochondria-mediated apoptosis[12]. However, the condition of Apaf-1 gene expression in gastric cancer and the correlation between expression of Apaf-1 gene and genesis of gastric cancer remain unclear. Although Apaf-1 is an important apoptosis factor and a tumor suppressor gene[12-16], few studies on the relationship between Apaf-1 gene and gastric cancer are available.

In the present study, Apaf-1 gene expression in gastric cancer tissue samples was significantly lower than that in adjacent normal tissue samples, suggesting that Apaf-1 gene plays an important role in genesis and progression of gastric cancer.

According to the epigenetics theory on gene regulation, methylation of gene fragments (especially promoter domain) inhibits gene transcription, and acetylation of gene-related histone up-regulates gene expression. One of the carcinogenic mechanisms is gene silencing caused by hyper-methylation of CpG islet at tumor suppressor gene promoter domain and deacetylation of histone[17-23]. It was reported that Apaf-1 gene is a tumor suppressor gene and seldom mutates, but has functional loss due to LOH and promoter methylation[24]. Apaf-1 gene has different effects and expression patterns in cancer tissue of different sources. The lower (or inactive) expression of Apaf-1 gene is related to methylation silencing in acute leukemia and laryngeal squamous carcinoma[24,25], and LOH in colon cancer, ovarian cancer and malignant melanocarcinoma[26-28].

In the present study, Apaf-1 gene promoter was methylated in 14 gastric cancer tissue samples with a decreased expression of Apaf-1 as detected by the MSP technique. Apaf-1 expression decreased in 13 gastric cancer tissue samples with LOH at more than 2 sites (including promoter methylation in 6 cases) as demonstrated by 9% non-denaturing PAGE. These findings show that decreased expression of Apaf-1 gene in gastric cancer tissue is due to some complex reasons, among which, however, promoter methylation and LOH at polymorphic sites play a major role.

COMMENTS

Background

The expression condition of Apaf-1 gene in gastric cancer and its correlation with the genesis of gastric cancer remain unclear. The purpose of this study was to make it clear.

Research frontiers

The peigenetics theory on gene regulation is the highlight in recent tumor studies. This article describes the role of Apaf-1 gene eigenetics in gastric cancer.

Innovations and breakthroughs

This article focuses on the Apaf-1 expression condition in gastric cancer, and its relation with loss of heterozygosity and methylation in promoter domain.

Applications

Apaf-1 gene is an important apoptosis gene. Its status of methylation and LOH may contribute to the study on the etiology of gastric cancer.

Terminology

Methylation is a term used in chemical sciences to denote the attachment or substitution of a methyl group on various substrates. DNA methylation profiling is gaining momentum as an epigenetic approach to understanding the effects of aberrant methylation (either hyper- or hypomethylation) both in basic research and in clinical applications.

Peer review

Low expression of Apaf-1 gene in gastric cancer tissue was shown in this study, indicating that methylation of Apaf-1 gene promoter and LOH in domain of 12q22-23 are the main reasons for expression and altered expression of Apaf-1 gene in gastric cancer

REFERENCES

1 **Mitsunaga M**, Tsubota A, Nariai K, Namiki Y, Sumi M, Yoshikawa T, Fujise K. Early apoptosis and cell death induced by ATX-S10Na (II)-mediated photodynamic therapy are Bax- and p53-dependent in human colon cancer cells. *World J Gastroenterol* 2007; **13**: 692-698

2 **Wang X**, Ye Z, Zhong J, Xiang J, Yang J. Adenovirus-mediated Il-24 expression suppresses hepatocellular carcinoma growth via induction of cell apoptosis and cycling arrest and reduction of angiogenesis. *Cancer Biother Radiopharm* 2007; **22**: 56-63

3 **Chiang CT**, Way TD, Lin JK. Sensitizing HER2-overexpressing cancer cells to luteolin-induced apoptosis through suppressing p21(WAF1/CIP1) expression with rapamycin. *Mol Cancer Ther* 2007; **6**: 2127-2138

4 **Leo C**, Richter C, Horn LC, Schütz A, Pilch H, Höckel M. Expression of Apaf-1 in cervical cancer correlates with lymph node metastasis but not with intratumoral hypoxia. *Gynecol Oncol* 2005; **97**: 602-606

5 **Mustika R**, Budiyanto A, Nishigori C, Ichihashi M, Ueda M. Decreased expression of Apaf-1 with progression of melanoma. *Pigment Cell Res* 2005; **18**: 59-62

6 **Ekert PG**, Read SH, Silke J, Marsden VS, Kaufmann H, Hawkins CJ, Gerl R, Kumar S, Vaux DL. Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol* 2004; **165**: 835-842

7 **Anichini A**, Mortarini R, Sensi M, Zanon M. APAF-1 signaling in human melanoma. *Cancer Lett* 2006; **238**: 168-179

8 **Ho CK**, Bush JA, Li G. Tissue-specific regulation of Apaf-1 expression by p53. *Oncol Rep* 2003; **10**: 1139-1143

9 **Sturm I**, Bosanquet AG, Radetzki S, Hummel M, Dörken B, Daniel PT. Silencing of APAF-1 in B-CLL results in poor prognosis in the case of concomitant p53 mutation. *Int J Cancer* 2006; **118**: 2329-2336

10 **Marsden VS**, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, Roy S, Nicholson DW, Vaux DL, Bouillet P, Adams JM, Strasser A. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* 2002; **419**: 634-637

11 **Sobin LH**, Fleming ID. TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997; **80**: 1803-1804

12 **Zou H**, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; **90**: 405-413

13 **Zlobec I**, Minoo P, Baker K, Haegert D, Khetani K, Tornillo L, Terracciano L, Jass JR, Lugli A. Loss of APAF-1 expression is associated with tumour progression and adverse prognosis in colorectal cancer. *Eur J Cancer* 2007; **43**: 1101-1107

14 **Deng WG**, Kawashima H, Wu G, Jayachandran G, Xu K, Minna JD, Roth JA, Ji L. Synergistic tumor suppression by coexpression of FUS1 and p53 is associated with down-regulation of murine double minute-2 and activation of the apoptotic protease-activating factor 1-dependent apoptotic pathway in human non-small cell lung cancer cells. *Cancer Res* 2007; **67**: 709-717

15 **Christoph F**, Kempkensteffen C, Weikert S, Köllermann J, Krause H, Miller K, Schostak M, Schrader M. Methylation of tumour suppressor genes APAF-1 and DAPK-1 and in vitro effects of demethylating agents in bladder and kidney cancer. *Br J Cancer* 2006; **95**: 1701-1707

16 **Zlobec I**, Steele R, Michel RP, Compton CC, Lugli A, Jass JR. Scoring of p53, VEGF, Bcl-2 and APAF-1 immunohistochemistry and interobserver reliability in colorectal cancer. *Mod Pathol* 2006; **19**: 1236-1242
17 **Roman-Gomez J**, Jimenez-Velasco A, Barrios M, Prosper F, Heiniger A, Torres A, Agirre X. Poor prognosis in acute lymphoblastic leukemia may relate to promoter hypermethylation of cancer-related genes. *Leuk Lymphoma* 2007; **48**: 1269-1282
18 **Ye M**, Xia B, Guo Q, Zhou F, Zhang X. Association of diminished expression of RASSF1A with promoter methylation in primary gastric cancer from patients of central China. *BMC Cancer* 2007; **7**: 120
19 **Karpf AR**. Epigenomic reactivation screening to identify genes silenced by DNA hypermethylation in human cancer. *Curr Opin Mol Ther* 2007; **9**: 231-241
20 **Lindsey JC**, Lusher ME, Anderton JA, Gilbertson RJ, Ellison DW, Clifford SC. Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma. *Br J Cancer* 2007; **97**: 267-274
21 **Ren Y**, Liu X, Ma D, Feng Y, Zhong N. Down-regulation of the progesterone receptor by the methylation of progesterone receptor gene in endometrial cancer cells. *Cancer Genet Cytogenet* 2007; **175**: 107-116
22 **Watanabe T**, Katayama Y, Yoshino A, Yachi K, Ohta T, Ogino A, Komine C, Fukushima T. Aberrant hypermethylation of p14ARF and O6-methylguanine-DNA methyltransferase genes in astrocytoma progression. *Brain Pathol* 2007; **17**: 5-10
23 **Nelson WG**, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM. Abnormal DNA methylation, epigenetics, and prostate cancer. *Front Biosci* 2007; **12**: 4254-4266
24 **Huang DF**, Fu WN, Shang C, Xu ZM, Li ZG, Sun KL. Expression and promoter methylation of Apaf-1 gene in laryngeal squamous cell carcinoma. *Yichuan Xuebao* 2004; **31**: 1327-1331
25 **Fu WN**, Bertoni F, Kelsey SM, McElwaine SM, Cotter FE, Newland AC, Jia L. Role of DNA methylation in the suppression of Apaf-1 protein in human leukaemia. *Oncogene* 2003; **22**: 451-455
26 **Murty VV**, Montgomery K, Dutta S, Bala S, Renault B, Bosl GJ, Kucherlapati R, Chaganti RS. A 3-Mb high-resolution BAC/PAC contig of 12q22 encompassing the 830-kb consensus minimal deletion in male germ cell tumors. *Genome Res* 1999; **9**: 662-671
27 **Umetani N**, Fujimoto A, Takeuchi H, Shinozaki M, Bilchik AJ, Hoon DS. Allelic imbalance of APAF-1 locus at 12q23 is related to progression of colorectal carcinoma. *Oncogene* 2004; **23**: 8292-8300
28 **Baldi A**, Santini D, Russo P, Catricalà C, Amantea A, Picardo M, Tatangelo F, Botti G, Dragonetti E, Murace R, Tonini G, Natali PG, Baldi F, Paggi MG. Analysis of APAF-1 expression in human cutaneous melanoma progression. *Exp Dermatol* 2004; **13**: 93-97

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