PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

ELSEVIER

World J Gastroenterol 2005;11(31):4852-4856
World Journal of Gastroenterology ISSN 1007-9327

• *BRIEF REPORTS* •

Overexpression of S100A4 is closely associated with progression of colorectal cancer

Yong-Gu Cho, Chang-Jae Kim, Suk-Woo Nam, Shin-Hee Yoon, Sug-Hyung Lee, Nam-Jin Yoo, Jung-Young Lee, Won-Sang Park

Yong-Gu Cho, Chang-Jae Kim, Suk-Woo Nam, Sug-Hyung Lee, Nam-Jin Yoo, Jung-Young Lee, Won-Sang Park, Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, South Korea
Shin-Hee Yoon, Department of Physiology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, South Korea
Supported by the Korea Science and Engineering Foundation, No. R13-2002-005-01004-0
Correspondence to: Dr. Won-Sang Park, Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, South Korea. wonsang@catholic.ac.kr
Telephone: +82-2-590-1192 Fax: +82-2-537-6586
Received: 2005-01-11 Accepted: 2005-01-26

Abstract

AIM: To investigate whether S100A4 played an important role in the development or progression of colorectal cancer.

METHODS: A total of 124 colorectal adenocarcinoma tissue specimens were analyzed by immunohistochemistry for the expression of S100A4 protein and subsequently investigated for the gene mutations in the coding region of *S100A4* gene. The specimens were collected over a 3-year period in the laboratories at our large teaching hospital in Seoul, Republic of Korea.

RESULTS: Normal colonic epithelium either failed to express or showed focal weak expression of S100A4. Moderate to strong cytoplasmic expression of S100A4 was seen in 69 (55.6%) of the 124 colorectal carcinoma tissue specimens. S100A4 expression was detected in 43 (69.4%) of 62 specimens with lymph node metastasis. Statistically, overexpression of S100A4 was significantly associated with Dukes' stage and lymph node metastasis. Nuclear staining was also observed in 24 (19.4%) of 124 samples and closely associated with Dukes' stage. However, there was no significant correlation between overexpression of S100A4 and other investigated clinico-pathologic parameters, including tumor localization, tumor size, and survival period. In mutational analysis, no gene mutation was found in the analyzed genomic area of colorectal cancer.

CONCLUSION: Overexpression of S100A4 may be closely related with the aggressiveness of colorectal carcinoma.

Key words: S100A4; Mutation; Immunohistochemistry; Colorectal cancer; Tumor stage

Cho YG, Kim CJ, Nam SW, Yoon SH, Lee SH, Yoo NJ, Lee JY, Park WS. Overexpression of S100A4 is closely associated with progression of colorectal cancer. *World J Gastroenterol* 2005; 11(31): 4852-4856
http://www.wjgnet.com/1007-9327/11/4852.asp

INTRODUCTION

Colorectal cancer remains one of the most frequent malignant neoplasms worldwide. In Korea, it accounts for an estimated 9.9% of all malignancies, with 9.7% in the male population and 10.2% in the female population[1]. The major cause of death is the metastatic spread of the disease from the primary tumor to distant sites, especially to the liver[2]. Research into colorectal cancer has highlighted the prognostic significance of TNM tumor staging including depth of tumor invasion, involvement of regional lymph nodes, and infiltration to distant organs. Although there are a lot of reports showing the significance of many other prognostic parameters such as histological grade, serum carcino-embryonic antigen levels and flow cytometric DNA analysis, none of them has been widely used in the clinic[3-5].

The concept of multi-stage carcinogenesis has been widely accepted as a consequence of multiple genetic alterations accumulated in cancer cells[6]. Interestingly, stepwise accumulation of genetic alterations during progression has observed in several tumor types, particularly in tumors of epithelial origin like colorectal cancer. It is also well known that highly metastatic cells often acquire more genetic alterations than non-metastatic cells. Therefore, it is indispensable to identify the genes, whose alterations accumulate during cancer progression as well as the genes, whose expression is responsible for the acquisition of invasive and/or metastatic potential in cancer cells.

The S100 family of calcium binding proteins has been shown to be involved in a variety of physiological functions, such as cell proliferation, extracellular signal transduction, intercellular adhesion, and motility as well as cancer metastasis[7-9]. Of these, S100A4 (mts1, p9Ka, calvasculin) has been identified as a cytoplasmic protein in normal cells, which is associated with the actin/myosin cytoskeleton in fixed cells[10]. Interestingly, elevated levels of S100A4 are closely associated with the process of metastasis in several human solid cancers including gastric cancer[11,12], colorectal adenocarcinoma[13-15], and breast cancer[16]. Recently,

Flatmark *et al*.[15], reported, that nuclear localization of S100A4 is correlated with tumor stage in colorectal cancer. Furthermore, S100A4 secreted from tumor cells can increase endothelial cell motility and hence induce angiogenesis[17]. All these findings suggest that S100A4 may exert its effect on metastasis formation not only by stimulating the motility of tumor cells but also by affecting their invasive properties through deregulation of the extracellular matrix[18]. In the present study, to investigate whether S100A4 played an important role in the development and/or progression of Korean colorectal cancers, the expression patterns of S100A4 in 124 colorectal adenocarcinoma tissues were examined. We also performed mutational analysis of the *S100A4* gene, one of the possible overexpression mechanisms of oncogenic proteins.

MATERIALS AND METHODS

Tissue samples

One hundred and twenty-four colorectal cancer patients between 2001 and 2002 were enrolled in this study and their tissue samples were formalin-fixed and paraffin-embedded. No patient had a family history of colorectal cancer. Tumor stage was classified according to Dukes' criteria. Thirteen patients were classified as Dukes' A, 47 as Dukes' B, 56 as Dukes' C and 8 as Dukes' D. The observation time was 14-38 mo for the survivors. Among the 113 patients who were followed up, 15 patients showed relapse of cancer and 11 patients died of cancer during this time. Two pathologists screened histological sections and selected areas of the representative tumor cells. Three tissue cores (0.6 mm in diameter) were taken from each tumor sample and placed in a new recipient paraffin block using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD, USA), according to the established methods[19]. One cylinder of normal colonic mucosa adjacent to each tumor was also transferred to the recipient block.

Microdissection

The histological section were stained with hematoxylin and eosin (H and E) and reviewed. Malignant cells were selectively procured from H and E stained slides without normal cell contamination using a laser micro-dissection device (ION LMD, Jungwoo International Co, Seoul, South Korea). Corresponding normal cells were obtained from non-metastatic lymph nodes. DNA was extracted by a modified single-step DNA extraction method, as described previously[20].

Single strand conformation polymorphism (SSCP) analysis

Genomic DNAs from tumor cells and corresponding normal cells were amplified with 2 primer pairs covering exons 2 and 3, the coding region of *S100A4*. The primer sequences were as follows: 5'-CCAGATCCTGACTGCT GTC-3' and 5'-GACTCACTCAGGCACTACCC-3' for exon 2, and 5'-GGGCTTCTGTTTTCTATC TGT-3' and 5'-CCAACCACA TCAGAG GAG-3' for exon 3. Each PCR was performed under standard conditions in a 10 μL reaction mixture containing 1 μL of template DNA, 0.5 μmol/L of each primer, 0.2 μmol/L of each deoxynucleotide triphosphate, 1.5 mmol/ L $MgCl_2$, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer, Foster City, CA, USA), 0.5 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, UK), and 1 μL of 10X buffer. The reaction mixture was denatured for 1 min at 94 ℃ and amplified for 35 cycles (denaturing for 40 s at 94 ℃, annealing for 40 s at 56 ℃, and extending for 40 s at 72 ℃). Final extension was continued for 5 min at 72 ℃. After amplification, PCR products were denatured for 5 min at 95 ℃ at a 1:1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH and loaded onto a SSCP gel (FMC mutation detection enhancement system; Intermountain Scientific, Kaysville, UT, USA) with 10% glycerol. After electrophoresis, the gels were transferred to 3-mm Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). We repeated the experiment thice, including tissue micro-dissection, PCR, SSCP, and sequencing, and found that the data were consistent.

Immunohistochemistry for S100A4

The primary polyclonal rabbit anti-S100A4 antibody (DAKO, Carpinteria, CA, USA, dilution 1/200) was used. Immunostaining was performed on microarray tissue sections with a tyramide signal amplification kit (NEN Life Science, Boston, MA, USA) for signal intensification. Antigen retrieval was performed by microwave heating in a citrate buffer (pH 6.0). Other procedures were performed as previously described[21]. The reaction products were developed with diaminobenzidine (Sigma, St Louis, MO, USA) and counterstained with hematoxylin. As a negative control, we used non-immune rabbit serum instead of the S100A4 antibody. Three pathologists independently reviewed the results. For statistical analysis, the stained sections were scored microscopically. The number of tumor cells stained in the cytoplasm was semi-quantitatively estimated and classified into negative and positive: negative $0 \leqslant 30\%$ and positive $\geqslant 30\%$ labeling in tumor cells.

RESULTS

Mutational analysis

We analyzed mutations of the *S100A4* gene in 124 colorectal carcinoma tissue specimens. There was no aberrant SSCP pattern in DNAs extracted from cancer cells, suggesting that there were no somatic mutations in the coding regions of the *S100A4* gene in colorectal carcinoma. We found a single nucleotide polymorphism, which was an A to G transition at nucleotide number 99 in both corresponding normal and tumor DNAs of cases No. 10 and No. 67 (data not shown). The variation was an identical single nucleotide polymorphism found in our previous report[11] and showed no amino acid change at codon 33 (Glu→Glu, GAA→GAG). The data were consistent with triplicate experiments.

Expression of S100A4

One hundred and twenty-four colorectal carcinoma tissue specimens were screened for S100A4 protein expression. The expression was mainly faint or negative in normal colonic

Table 1 Relationship between expression of S100A4 and tumor stage of colorectal carcinoma

	Cytoplasm		Positive (%)	*P*	Nuclear		Positive (%)	*P*
	+	-			+	-		
Stage				0.0001[1]				<0.05[2]
A	4	9	30.8		1	12	7.7	
B	21	26	44.6		7	40	14.9	
C	37	19	66.1		12	44	21.4	
D	7	1	87.5		4	4	50.0	
L/N metastasis				0.0255[3]				0.5184[3]
+	39	20	66.1		10	49	16.9	
-	30	35	46.1		14	51	21.5	
Site				0.5154[3]				0.2719[3]
Right	12	14	46.2		7	19	26.9	
Left	57	41	53.4		17	81	17.3	
Tumor size				0.6421[3]				1.0000[3]
<5 cm	33	24	57.9		11	46	19.2	
≥5 cm	36	31	53.7		13	54	19.4	
Survival period				0.7719[3]				0.9563[3]
<24 mo	6	4	60.0		2	8	20.0	
≥24 mo	63	51	55.3		22	92	19.3	
Total	69	55			24	100		

[1]Cochran's linear trend test; [2]Bartholomew test; [3]χ^2 test.

mucosa, but moderate to strong in lymphocytes and smooth muscle cells, concordant with previous report[14]. In the present study, overexpression of S100A4 was found in 69 (55.6%) of the 124 colorectal carcinoma tissue specimens, in which immunostaining was predominantly marked on the cytoplasm of tumor cells (Figure 1). Cytoplasmic staining was seen in 30.8% (4 of 13) stage A cases, 44.6% (21 of 47) stage B cases, 66.1% (37 of 56) stage C cases, and 87.5% (7 of 8) stage D cases, respectively (Table 1). Statistically, overexpression of S100A4 was closely associated with Dukes' stage ($P<0.01$) and lymph node metastasis ($P<0.01$). However, there was no significant correlation between over-expression of S100A4 and other investigated clinico-pathologic parameters, including tumor localization, tumor size, and survival period (Table 1). Interestingly, 12 of 15 patients with recurrence of cancer demonstrated cytoplasmic staining at diagnosis. Nine of them died of cancer and 2 died of cardio-vascular disease.

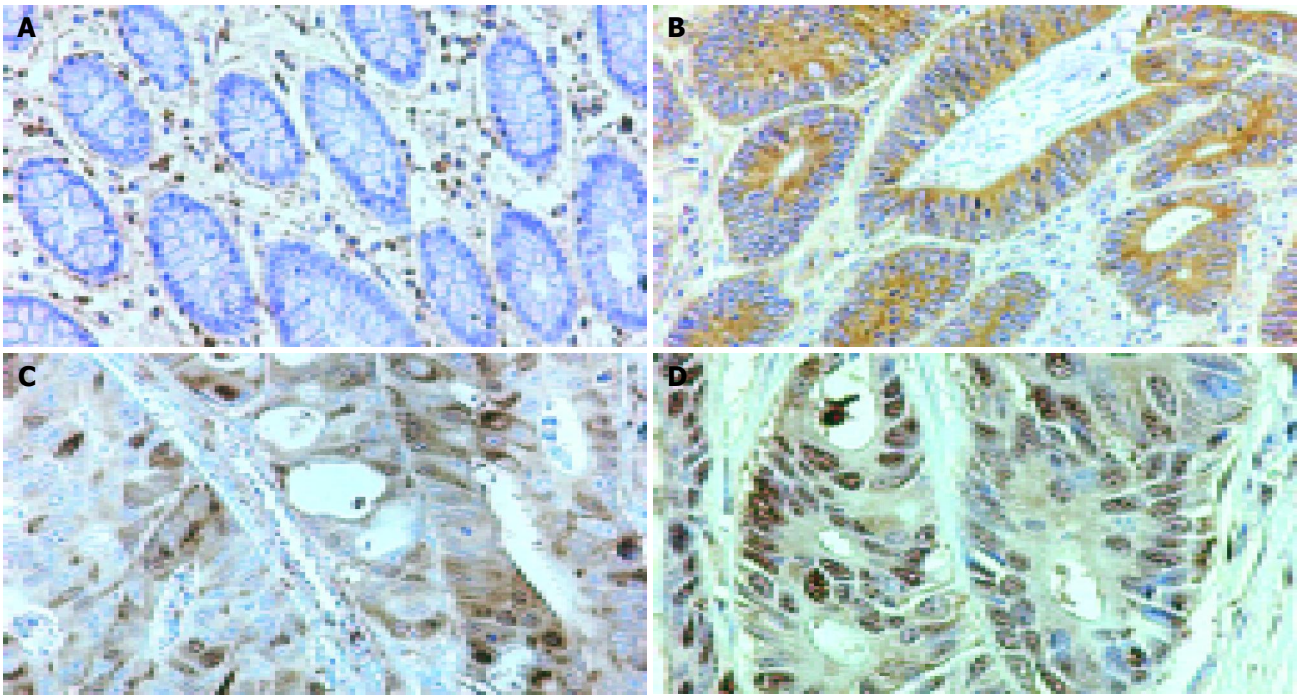

Figure 1 Expression of S100A4 in colonic mucosa (**A**), tubular adenocarcinoma (**B**, **C**), and nuclear staining of S100A4 (**D**). (Original magnifications: **A**-**C**, ×200; **D**, ×400).

Nuclear staining was also observed in 24 (19.4%) of 124 samples and the percentage of S100A4 positive cases was closely associated with Dukes' stage ($P<0.05$, Table 1). However, there was no correlation between nuclear staining of S100A4 and pathologic parameters, including lymph node metastasis, tumor localization, tumor size, and survival period (Table 1). Additionally, nuclear staining was found in 4 of 15 patients with relapse and 3 of them died of cancer.

DISCUSSION

Oncogene amplification usually occurs late in tumor progression and correlates well with clinical aggressiveness of tumors[21]. Over-expression of S100A4 has been reported in several human cancers, including gastric[11,12], colorectal[13-15], and breast cancers[16]. Recently, it has been suggested that nuclear localization of S100A4 is related to tumor stage of colorectal cancer, and S100A4 may be involved in gene regulatory pathways related to the metastatic phenotype of cancer cells[15].

In the present study, cytoplasmic over-expression of S100A4 was found in 69 (55.6%) of the 124 colorectal adenocarcinoma tissue specimens. Interestingly, the cytoplasmic expression of S100A4 was statistically associated with Dukes' stage and lymph node metastasis (Table 1). Additionally, 12 of 15 patients with recurrence of cancer demonstrated cytoplasmic staining and 9 of them died of cancer. It was reported that over-expression of S100A4 is closely correlated with a number of factors for tumor aggressiveness, such as lymph node metastasis, depth of invasion, and peritoneal dissemination[11]. Overexpression of S100A4 is more frequently found in cancer cells than in normal colonic mucosa, as well as more in liver metastasis than in primary tumors[14,15]. Furthermore, S100A4 expression has been proved to be a highly significant and independent prognostic marker in colorectal cancer[22]. These data further support the significant correlation between over-expression of S100A4 and progression of colorectal cancer, and the putative role of S100A4 in tumor cell aggressiveness [3,6,15].

In this study, nuclear staining of S100A4 was seen in 24 (19.4%) of 124 samples (Figure 1) and showed a significant association with higher Dukes' stage (Table 1). Previously, Flatmark *et al*.[15], examined the nuclear expression of S100A4 in colorectal cancer and reported that nuclear location of S100A4 is associated with tumor stage. Our results also suggest that nuclear translocation of S100A4 protein might be involved in the process of invasion and metastasis of colorectal cancer. It is possible that S100A4 regulates transcription of other genes either through direct DNA binding to or through interaction with other DNA-binding proteins. Further large-scale and functional studies are necessary to elucidate the effect of nuclear translocation of S100A4 on the progression of human cancers, including colorectal cancer.

Generally, activation of a proto-oncogene results from mutation, rearrangement or manifold amplification of the DNA sequences, like *N-myc* in neuroblastoma and *c-erb B2* in breast cancer[23-25]. Since there was no detectable somatic mutation of *S100A4* gene in the primary tumors in this study, we considered that the S100A4 over-expression might not result from genetic mutation in colorectal carcinogenesis. However, we cannot completely rule out the possibility of a genetic alteration in other regions, such as the promoter, non-coding exon, and splice sites. Another possibility is the amplification or hypo-methylation of the *S100A4* gene in colorectal cancer, as in pancreatic ductal adenocarcinoma[26]. In addition, our results may underestimate the prevalence of *S100A4* somatic mutations in colorectal cancer, as the sensitivity rate of SSCP analysis for the detection of single base substitutions is about 80%[27].

In conclusion, S100A4 is overexpressed in colorectal cancer, cytoplasmic and nuclear expression is closely associated with a number of factors for tumor aggressiveness, such as tumor stage and lymph node metastasis.

REFERENCES

1 **Suh CI,** Suh KA, Park SH, Chang HJ, Ko JW, Ahn DH. Annual report of the central cancer registry in Korea-1998. *J Korean Cancer Assoc* 2002; **32**: 827-834

2 **Soong R,** Grieu F, Robbins P, Dix B, Chen D, Parsons R, House A, Lacopetta B. p53 alterations are associated with improved prognosis in distal colonic carcinomas. *Clin Cancer Res* 1997; **3**: 1405-1411

3 **Hutter RV,** Sobin LH. A universal staging system for cancer of the colon and rectum. Let there be light. *Arch Pathol Lab Med* 1986; **110**: 367-378

4 **McLeod HL,** Murray GI. Tumour markers of prognosis in colorectal cancer. *Br J Cancer* 1999; **79**: 191-203

5 **Akbulut H,** Dincol D, Aydintug O, Icli F, Karaoguz H, Demirkazik A. The prognostic significance of flow cytometric DNA content determination in patients with colorectal carcinoma. *Turk J Cancer* 1998; **28**: 51-58

6 **Fearon ER,** Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767

7 **Schafer BW,** Heizmann CW. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci* 1996; **21**: 134-140

8 **Sherbet GV,** Lakshmi MS. S100A4 (MTS1) calcium binding protein in cancer growth, invasion and metastasis. *Anticancer Res* 1998; **18**: 2415-2422

9 **Davies BR,** Davies MP, Gibbs FE, Barraclough R, Rudland PS. Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial cell line with the gene for p9Ka, a rat calcium-binding protein, but not with the oncogene EJ-ras-1. *Oncogene* 1993; **8**: 999-1008

10 **Gibbs FEM,** Barraclough R, Platt-Higgins A, Rudland P, Wilkinson MC, Parry EW. Immunohistochemical distribution of the calcium-binding protein p9Ka in normal rat tissue: variation in the cellular location in different tissues. *J Histochem Cytochem* 1995; **43**: 169-180

11 **Cho YG,** Nam SW, Kim TY, Kim YS, Kim CJ, Park JY, Lee JH, Kim HS, Lee JW, Park CH, Song YH, Lee SH, Yoo NJ, Lee JY, Park WS. Overexpression of S100A4 is closely related to the aggressiveness of gastric cancer. *APMIS* 2003; **111**: 539-545

12 **Yonemura Y,** Endou Y, Kimura K, Fushida S, Bandou E, Taniguchi K, Kinoshita K, Ninomiya I, Sugiyama K, Heizmann CW, Schafer BW, Sasaki T. Inverse expression of S100A4 and E-cadherin is associated with metastatic potential in gastric cancer. *Clin Cancer Res* 2000; **6**: 4234-4242

13 **Taylor S,** Herrington S, Prime W, Rudland PS, Barraclough R. S100A4 (p9Ka) protein in colon carcinoma and liver metastases: association with carcinoma cells and T-lymphocytes. *Br J Cancer* 2002; **86**: 409-416

14 **Takenaga K,** Nakanishi H, Wada K, Suzuki M, Matsuzaki O, Matsuura A, Endo H. Increased expression of S100A4, a metastasis-associated gene, in human colorectal adenocarcinomas. *Clin Cancer Res* 1997; **3**: 2309-2316

15 **Flatmark K,** Pedersen KB, Nesland JM, Rasmussen H, Aamodt G, Mikalsen SO, Bjornland K, Fodstad O, Mælandsmo GM. Nuclear localization of the metastasis-related protein S100A4 correlates with tumour stage in colorectal cancer. *J Pathol* 2003; **200**: 589-595
16 **Rudland PS,** Platt-Higgins A, Renshaw C, West CR, Winstanley JH, Robertson L, Barraclough R. Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. *Cancer Res* 2000; **60**: 1595-1603
17 **Ambartsumian N,** Klingelhofer J, Grigorian M, Christensen C, Kriajevska M, Tulchinsky E, Georgiev G, Berezin V, Bock E, Rygaard J, Cao R, Cao Y, Lukanidin E. The metastasis-associated Mts1 (S100A4) protein could act as an angiogenic factor. *Oncogene* 2001; **20**: 4685-4695
18 **Bjørnland K,** Winberg JO, Odegaard OT, Hovig E, Loennechen T, Aasen AO, Fodstad O, Maelandsmo GM. S100A4 involvement in metastasis: deregulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in osteosarcoma cells transfected with an anti-S100A4 ribozyme. *Cancer Res* 1999; **59**: 4702-4708
19 **Kononen J,** Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847
20 **Lee JY,** Dong SM, Kim SY, Yoo NJ, Lee SH, Park WS. A simple, precise and economical microdissection technique for analysis of genomic DNA from archival tissue sections. *Virchows Arch* 1998; **433**: 305-309
21 **Park WS,** Oh RR, Kim YS, Park JY, Shin MS, Lee HK, Lee SH, Yoo NJ, Lee JY. Absence of mutations in the kinase domain of the Met gene and frequent expression of Met and HGF/SF protein in primary gastric carcinomas. *APMIS* 2000; **108**: 195-200
22 **Gongoll S,** Peters G, Mengel M, Piso P, Klempnauer J, Kreipe H, von Wasielewski R. Prognostic significance of calcium-binding protein S100A4 in colorectal cancer. *Gastroenterology* 2002; **123**: 1478-1484
23 **Yokota J,** Tsunetsugu-Yokota Y, Battifora H, Le Fevre C, Cline MJ. Alterations of myc, myb and ras^{Ha} proto-oncogenes in cancers are frequent and show clinical correlation. *Science* 1986; **231**: 261-265
24 **Brodeur GM,** Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121-1124
25 **Slamon DJ,** Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A. Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; **244**: 707-712
26 **Rosty C,** Ueki T, Argani P, Jansen M, Yeo CJ, Cameron JL, Hruban RH, Goggins M. Overexpresion of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. *Am J Pathol* 2002; **160**: 45-50
27 **Sheffield VC,** Beck JS, Kwitek AE, Sandstrom DW, Stone EM. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 1993; **16**: 325-332

Science Editor Wang XL and Guo SY **Language Editor** Elsevier HK