Overexpression of S100A4 is closely associated with progression of colorectal cancer

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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.

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Flatmark et al., 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. 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. 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**

**Tissues 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. 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.

**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'–CCAGATCTCTGAGCTGTC-3' and 5'–GACTCACTGCTAGGCAACG-3' for exon 2, and 5'–GGGCTTCTGTGTCTTTCATC-3' and 5'–CCAACCACAGCATGAGG-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 deoxynucleotide triphosphate, 1.5 mmol/L MgCl₂, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer, Foster City, CA, USA), 0.5 µCi of [³²P]dCTP (Amersham, Buckinghamshire, UK), and 1 µL of 10X buffer. The reaction mixture was denatured for 1 min at 94 °C and amplified for 35 cycles (denaturing for 40 s at 94 °C, annealing for 40 s at 56 °C, and extending for 40 s at 72 °C). Final extension was continued for 5 min at 72 °C. After amplification, PCR products were denatured for 5 min at 95 °C 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 Science, 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 thrice, 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. 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<30% and positive ≥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 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
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.

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

| Stage | Cytoplasm Positive (%) | P | Nuclear Positive (%) |
|-------|------------------------|---|----------------------|
| A     | 4 (30.8)               |   | 1 (7.7)              |
| B     | 21 (44.6)              | 7 | 40 (14.9)            |
| C     | 37 (66.1)              | 12| 44 (21.4)            |
| D     | 7 (87.5)               | 4 | 4 (50.0)             |

| L/N metastasis | Cytoplasm Positive (%) | P | Nuclear Positive (%) |
|----------------|------------------------|---|----------------------|
| +              | 39 (66.1)              | 10| 49 (16.9)            |
| -              | 30 (46.1)              | 14| 51 (21.5)            |

| Site | Cytoplasm Positive (%) | P | Nuclear Positive (%) |
|------|------------------------|---|----------------------|
| Right| 12 (46.2)              | 7 | 19 (26.9)            |
| Left | 57 (53.4)              | 17| 81 (17.3)            |

| Tumor size | Cytoplasm Positive (%) | P | Nuclear Positive (%) |
|------------|------------------------|---|----------------------|
| <5 cm      | 33 (57.9)              | 11| 46 (19.2)            |
| ≥5 cm      | 36 (53.7)              | 13| 54 (19.4)            |

| Survival period | Cytoplasm Positive (%) | P | Nuclear Positive (%) |
|-----------------|------------------------|---|----------------------|
| <24 mo          | 6 (60.0)               | 2 | 8 (20.0)             |
| ≥24 mo          | 63 (55.3)              | 22| 92 (19.3)            |
| Total           | 69 (98.7)              | 24| 100 (100)            |

1Cochran’s linear trend test; 2Bartholomew test; 3$\chi^2$ test.

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[23]. 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[6,4,9,18].

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.[13], 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[21-25]. 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.

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