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ÖZGÜN MAKALE/ORIGINAL ARTICLE

CLINICAL AND PATHOLOGICAL SIGNIFICANCE OF S100A4 EXPRESSION IN COLON CANCER

Kolon Kanserinde S100A4 Ekspresyonunun Klinik ve Patolojik Önemi

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ABSTRACT

Aim: Because the S100A4 gene is recognized as a marker in metastasis (in vitro, in vivo) and invasion, this study aimed to determine the clinical and pathological significance of S100A4 expression in patients with colorectal cancer.

Material and Methods: S100A4 expression was evaluated through samples taken from blocked resection material from 111 colon cancer patients and through immunohistochemical staining scores (IHCSS). We studied the relationships between IHCSS and patient age, gender, tumor localization, tumor diameter, surgical procedure, perineural invasion, perivascular invasion, T stage, N stage, M stage, disease stage, tumor grade, and metastatic lymph node ratio, comparing survival and mortality rates.

Results: No statistically significant difference was spotted when comparing IHCSS against patient gender, patient age, tumor localization, surgery performed, or tumor diameter. As for perivascular invasion, lymph node involvement, and degree of metastasis, all of which are considered to intensify tumor progression and aggressivity, each increased in direct proportion to IHCSS. As a result, because S100A4 expression influences tumor metastasis and invasion, an increase in S100A4 proteins may herald a poor prognosis.

Conclusion: Thus, S100A4 expression may be useful for determining more effective treatment strategies in colorectal cancer. Further studies are needed at the clinical level to confirm the practicality of using S100A4 expression to augment patient treatment.

Key words: Colon cancer, S100A4, calcium binding proteins

ÖZET

Amaç: Bu çalışma, invitro ve invivo metastaz ve invazyon belirteci olarak gösterilmiş olan S100A4 ekspresyonunun kolorektal kanserli hastalarda klinik ve patolojik önemini belirlemek amacıyla yapılmıştır.

Gereç ve Yöntem: 111 kolon kanserli hastanın bloklanmış rezeksiyon materyallerinden kesitler alınmış ve immunhistokimya boyama skorlaması (İHKBS) yapılarak S100A4 ekspresyonu değerlendirilmiştir. İHKBS ile hasta yaşı, cinsiyeti, tümör lokalizasyonu, tümör çapı, ameliyat tipi, perinöral invazyon varlığı, perivasküler invazyon varlığı, T evresi, N evresi, M evresi, hastalık evresi, tümör grade'i, metastatik lenf nodu yüzdesi, sağkalım ve mortalite arasındaki ilişki karşılaştırılmıştır.

Bulgular: Hasta cinsiyeti, hasta yaşı, tümör lokalizyonu, ameliyat tipi ve tümör çapı ile İHKBS arasında yapılan karşılaştırmalarda anlamlı bir istatiksel fark elde edilmedi. Tümör progresyonu ve agresivitesini arttıran perivasküler invazyon, perinöral invazyon, lenf nodu tutulumu ve metastaz parametrelerinin ise İHBS ile doğru orantılı olarak arttığı gözlenmiştir.

Sonuç: Sonuç olarak tümör metastazı ve invazyonunu artıran S100A4'nün ekspresyonundaki artışın kötü prognoz göstergesi olabileceği değerlendirilmiştir. Kötü prognostik faktörlerle ilişkili olan S100A4 ekspresyonunun kolorektal kanser tedavi stratejilerinin belirlenmesinde kullanılması mümkün olabilir. Bu konunun klinik pratiğe yansıtılması için daha ileri çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Kolon kanseri, S100A4, kalsiyum bağlayıcı proteinler

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INTRODUCTION

S100 proteins which are calcium binding proteins (CaBP) were firstly recognized by Moore and took their name as they are soluble in 100% amnion sulphate solution (1). S100 protein family consists of at least twenty one different proteins having common calcium binding EF-hand motif and its molecular weight varies from 9 to 13 kDa (2). S100 proteins play a significant role in various functions such as protein phosphorylation, immune response, growth, differentiation, cytoskeletal movement, enzyme activity and Ca2+ homeostasis by interacting with different target proteins. Extracellular S100 proteins have a part in neuronal differentiation, astrocyte proliferation by stimulating the activity of inflammatory cells (3,4). Overexpression of S100 proteins is observed in cardiac diseases, neurodegenerative and inflammatory diseases, psoriasis, wound healing and cancer especially such as melanoma(5). In the clinical trials made, it has been stated that S100A4 is a molecular marker related to clinical metastatic tumours and S100A8 and S100A9 are overexpressed in the early period of prostate cancer (6,7). S100B concentration in the circulation is also a marker for determining the direction of treatment in cancer such as melanoma (8).

Searching various medications ensuring that calcium concentration is kept at a certain level in cell by affecting CaBP and determining the roles of these medications in the treatment of some diseases are among the issues remaining to be searched and in our opinion, these are important research areas. A great many correlated molecular researches are made by clinic concerning colorectal cancer (CRC) and new markers to lead the treatment team especially in determining the course of disease are sought. New information to be obtained about the course of any type of CRCs is so important to properly choose the treatment alternatives and to develop new treatment strategies. In this study, it was aimed to reveal whether the expression of S1004A protein is associated with the tumour progression and metastasis characteristics in patients with colon cancer and to observe its prognostic significance in our clinic. The fact that the relationship of S100A4 protein enabling in vitro and in vivo invasion and metastasis with clinical and pathological characteristics of colon cancer was revealed and the value of this characteristic as a prognostic criterion were searched.

MATERIAL AND METHOD

This study was made by using recorded clinical data and the preparations prepared by paraffin-embedded blocks of patients with colon cancer treated in our clinic between the years of 1995 and 2008 by the approval of Gulhane Medical School local ethics committee (05.03.2010. 1491-688-10/1539). This is a retrospective study.

Total 111 patients, 67 of whom were males, 44 of whom were females were included in the study. Age, gender, tumour localization, M stage, surgery type, morality and survival times of patients were retrospectively obtained from patient files and hospital information system. Tumour size, T stage, N stage, metastatic lymph node percentage, grading of tumour, perivascular invasion, perineural invasion and stage were retrospectively collected from pathology reports. S100A4 immunohistochemical staining was made by preparing new preparations from paraffin-

embedded blocks and the staining score was calculated. For the evaluation of survival, all patients or their relatives were contacted and whether they were alive or not was determined.

Criteria for Exclusion from the Study: The patients whose clinical and pathological data determined for the study could not be reached and the patients who could not be followed and the patients who could not be contacted were excluded from the study. Additionally, the patients whose immunohistochemical staining technically failed were also excluded from the study. Rectum located cancers were excluded from the study to enable homogeneity in the evaluation of TNM stage. Death due to complications caused by surgery within 30 days after surgery or death before leaving hospital after initial hospital admission were accepted as criteria for exclusion from the study. Patients having extra colonic second primary tumour, the presence of synchronous or metachronous tumours and the presence of significant associated diseases resulting in the death of patient were evaluated as the reason why to be excluded from the study.

Sections will be stained with S100A4, xylene, and high concentration to quench endogenous peroxidase activity then deparaffinised alcohol were incubated in hydrogen peroxide solution. Then boiled in citrate buffer for antigen recovery purposes within was allowed to cool to room temperature. ultra block protein were incubated in blocking solution dropwise. Thereafter sections were stained with rabbit antibodies diluted 1/100 dropwise S100A4 was incubated at room temperature. The biotinylated Goat Anti-Polyvalent solution stood still dropwise onto the sections. sections were incubated in PBS wash buffer between steps. Streptavidin-peroxidase solution was added dropwise onto the sections were incubated again. DAB chromogen incubated with sections for hematoxylin counter stain was performed. Sections through alcohol, dried etuve and was sealed with Entellan.

All immunohistochemistry applied sections were examined by two independent observers. Cytoplasmic staining for S100A4 was evaluated by applying staining scoring system. A lymph node and melanoma section was taken as positive control. For negative control, staining that S100A4 showed on peripheral nerve was accepted as the strongest in the evaluation of immunohistochemical sections by dripping non-specific immune serum instead of primary antibodies and accordingly the staining density was determined as negative (0), weak (+1), medium (+2) and strong (+3). The percentage of cell stained was determined as 0-10% (1), 10-25% (2), 25-50% (3) and 50-100% (4). These two scores were multiplied by each other. Immunohistochemical staning score (IHCSS) was obtained as 1 for the ones having score 0 (Figure 1), as 2 for the ones having score between 1-4 (Figure 2), as 3 for the ones having score between 5-8 (Figure 3), as 4 for the ones having score 9-12 (Figure 4) (Table 1).

Statistical Evaluation: When evaluating the findings obtained from the study, “SPSS 15.0” package program was used for statistical analysis.

As for the comparison of qualitative data, Chi-square Test, Log-rank Test, Univariate Cox-regression Test and Multivariate Cox-regression Test were used. The fact that P value was less than 0.05 was accepted statistically significant.

RESULTS

Average of patient age was determined as 60.9 (21-90). No significant result was obtained from the comparison of the relationship between age and gender and IHCSS.

There were 6 patients having Grade 1 (well-differentiated), 72 patients having Grade 2 (moderately-differentiated) and 33 patients having Grade 3 (poorly-differentiated). No significant result was obtained from the comparison made between the Grade of tumour and IHCSS (Pearson Chi-square, P=0.751) (Table 2).

It was determined that as the number of metastatic lymph node and the percentage of metastatic lymph node rose, IHCSS increased (P=0.001). It was observed that 27 of 111 patients had perivascular invasion. It was also observed that the patients having perivascular invasion had higher IHCSS (Pearson Chi-square, P=0.022) (Table 3)

It was observed that 19 of 111 patients had perivascular invasion. It was also observed that the patients having perineural invasion had high IHCSS (Pearson Chi-square, P=0.023) (Table 4).

When T stages of the patients were evaluated, 1 of whom was at T1, 8 of whom were at T2, 11 of whom were at T3 and 91 of whom were at T4 stage. As for the evaluation of N stage, it was determined that 58 patients were as N0, 24 patients were as N1 and 29 patients were as N2. There were 85 patients having M0 stage and 26 patients having M1 stage. It was observed that as T stage, N stage and M stage increased, IHCSS rose. According to TNM staging, 8 of patients were at Stage I, 49 of whom were at Stage II and 26 of whom were at Stage IV. It was found that as the stages of tumours significantly progressed, their IHCSS were high (Pearson Chi-square, P<0.001) (Table 5).

Average follow-up period of patients was 44 months (2-113 months). It was seen that ss IHCSS increased, their 5-year survival rates decreased (Logrank test, P=0.002) (Table 6).

Figure 1: S100A4 expression at IHCSS 1 tumor (x200)

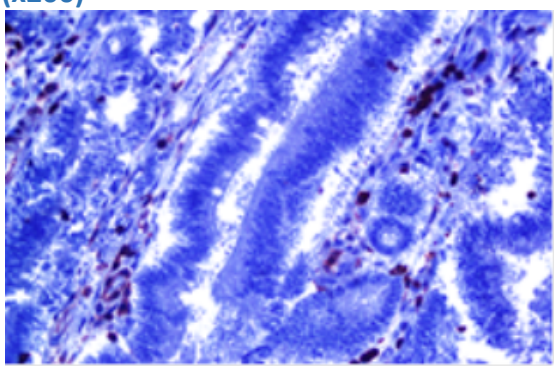


Figure 2: S100A4 expression at IHCSS 2 tumor (x200)

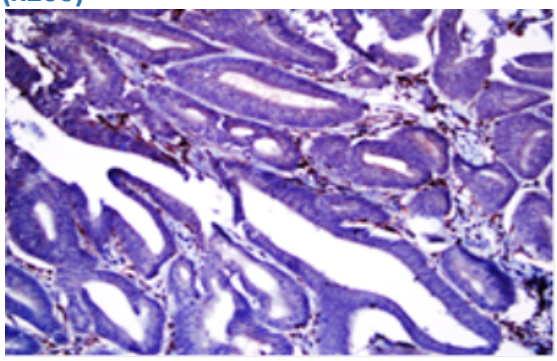
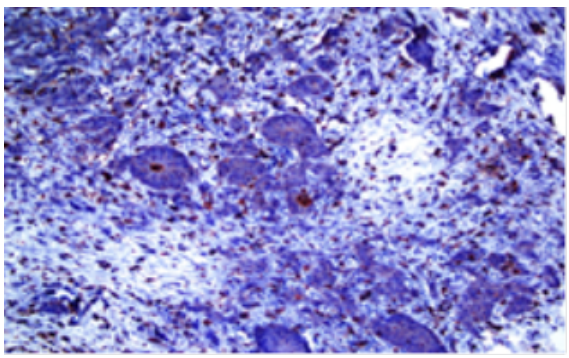


Figure 3: S100A4 expression at IHCSS 3 tumor (x200)



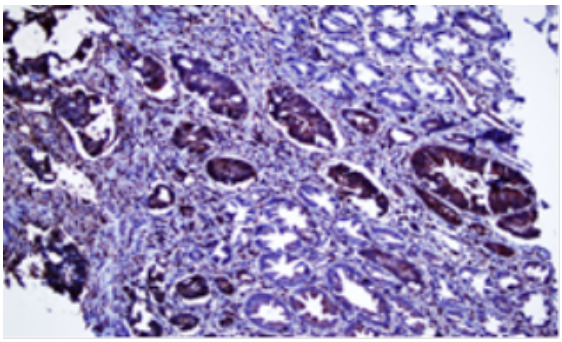


Figure 4: S100A4 expression at IHCSS 4 tumor (x200)

Table 1: Immunohistochemical staining scores

Staining intensity	Score
Negative	0
Weak	+1
Mild	+2
Strong	+3
Stained cell percentage	**Score**
0-10%	1
11-25%	2
26-50%	3
51-100%	4
IHCSS	**Total score (axb)**
1	0
2	1-4
3	5-8
4	9-12

Table 2: Relationship between the tumor grade and IHCSS.

HCSS	Grade 1	Grade2	Grade3	Total	P value
1	1	7	4	12	P=0.751
2	5	41	17	63	
3	0	19	10	29	
4	0	5	2	7	
Total	6(5.4%)	72(64%)	33(29.7%)	111	

No significant result was obtained from the comparison made between the Grade of tumour and IHCSS.

Table 3: Relationship between PVI (Perivascular invasion) and IHCSS.

IHCSS	PVI(-)	PVI(+)	P value
1	11	1	P=0.022
2	51	12	
3	16	13	
4	6	1	
Total	84(75.7%)	27(24.3%)	

It was also observed that the patients having perivascular invasion had higher IHCSS.

Table 4: Relationship between PNI (Perineural invasion) and IHCSS.

IHCSS	PNI(-)	PNI(+)	P value
1	12	0	P=0.023
2	55	8	
3	19	10	
4	6	1	
Total	92(%82.9)	19(%17.1)	

It was also observed that the patients having perineural invasion had high IHCSS.

Table 5: Relationship between IHCSS and tumor stage.

IHCSS	Stage I	Stage II	Stage III	Stage IV	P value
1	4	6	0	2	
2	3	40	13	7	
3	1	3	11	14	P<0.001
4	0	0	4	3	
Total	8(7.2%)	49(44.1%)	28(25.2%)	26(23. 5%)	

It was found that as the stages of tumours significantly progressed, their IHCSS were high.

Table 6: Relationship between IHCSS and 5 years survival rate.

IHCSS	N	5 years survival rate	P value
1	12	65.4%	
2	63	60.7%	P=0.002
3	29	21.8%	
4	7	57.1%	

It was seen that IHCSS increased, their 5-year survival rates decreased.

DISCUSSION

In various clinical studies, high level S100A4 expression is a significant factor for various cancer types (9-19) and metastasis in CRC (20-23) and appears as correlated with poor prognosis. S100A4 expression was shown in various cancer types such as breast, colorectal, stomach, medulloblastoma, prostate and bladder (24-29). As for our study, S100A4 overexpression in tumour cells in colon cancer was revealed by immunohistochemical staining. In the literature, the S100A4 positivity or IHCSS showing the overexpression of S100A4 or were calculated in different ways. In this study, when evaluating the immunohistochemical staining scores, the staining that S100A4 showed in peripheral nerve was accepted as the strongest and calculated. Accordingly, the staining density and the percentage of cell stained were determined. Both of these scores were multiplied by each other. Immunohistochemical staning score (IHCSS) was obtained as 1 for the ones having score 0, as 2 for the ones having score between 1-4, as 3 for the ones having score between 5-8, as 4 for the ones having score 9-12 (30). In a study made by Yong-Gu Cho et al. (31), the fact that the percentage of cell stained was higher than 30% was accepted as positive. In the scoring made by Ok-Jae Lee et al. (32), IHCSS was accepted as 0 for the ones having score 0, as 1for the ones having score 1-2, as 2 for the ones having score 2-6, as 3 for the ones having score 5-12, which were obtained from the multiplication of the staining density and the percentage of cell stained.

In advanced metastatic colon cancers, it was shown that S100A4 mRNA expression was higher than the ones which were non-metastatic (33). The metastatic activity of S100A4 which was overexpressed was shown in transgenic rats. In the studies made in various animal models, it was reported that S100A4 which did not affect the tumour progression enabled metastatic development (34,35). It was revealed that high S100A4 expression level was correlated with poor prognosis and low survival (36,37). As for our findings, when comparing the relationship between mortality and IHCSS, it was seen that as IHCSS increased, the mortality increased and the survival rates decreased.

In the studies made, it was shown that there was a correlation between lymph node involvement and S100A4 overexpression in CRCs (38). In our results, it was determined that as the number of metastatic lymph node and the percentage and metastatic lymph node increased, IHCSS increased. The fact that as the staining scores increased, the percentage of lymph node increased showed in parallel with

other literature information that S100A4 was active in invasion and metastasis.

If there is S100A4 expression in CRC, this increases the tumour progression and causes liver metastasis (21,25,39). In a study made in patients with non-metastatic colon cancer, it was observed that more metastasis presented in the follow-up of patients showing S100A4 expression (40). As for our study, when comparing the relationship with Pearson Chi-square test between T, N and M stages and IHCSS, all three were found statistically significant. As a result, it was observed that IHCSS was high in cases where T stage, N stage and M stage were high. It is not surprising that S100A4 overexpression showing the capacity of invasion and metastasis of tumour rather than tumorigenic effect occurs as T, N and M stages progress. When considering that perineural and perivascular invasions are poor prognostic factors, the fact that S100A4 was overexpressed in these cases revealed that S100A4 was a significant factor in terms of invasion and metastasis and had a prognostic value. The close relationship of S100A4 with these parameters determining the stage has a prognostic meaning. The fact that as the tumour stage known as a significant factor as prognostic value progressed, S100A4 expression got stronger became an important finding. Showing S100A4 expression in early affected patients is quite important when considering that it is an indicator that the tumour will gain aggressivity later. In the studies to be made for this subject, results to change our treatment strategies and to require personalized treatment protocols application may be obtained.

In this study, no significant result was determined in comparing the relationship between the grade of tumour and IHCSS. It was thought that this was related to the lack of number of grade 3 patients included in the study and the inhomogeneity of their grade distributions by staining scores.

In our study, basic factors affecting the prognosis and S100A4 overexpression were compared. Significant results were obtained between S100A4 and all other parameters excluding tumour differentiation and S100A4 was found as tumour aggressivity-increasing factor. However, S100A4 did not separately appear as a poor prognostic factor. As the reasons of this, factors such as the inadequacy of the number of patients examined and the inhomogeneity of patient distributions by stages can be listed. No significant statistical difference was obtained in the comparison made between gender of patient, age of patient, tumour localization, surgery type and tumour diameter and S100A4 overexpression evaluated by immunohistochemical staining. It was seen that S100A4 was independent from these parameters. The study made by Hemandas et al (41). showed parallelism with our study. In their studies, they could not reveal S100A4 expression as a separate independent prognostic factor and argued the inadequacy of the number of patient included in the study. Moreover, they pointed out that at least 12 lymph nodes should be excised in patients with stage III. Considering that in case the metastasis detection incidence of excision of more than 15 lymph nodes is 22% when they are excised by 85.15%; the fact that at least 12 lymph nodes are excised in each case is a factor to affect the treatment as well as affecting the stage. In their studies, they found the 5-year survival as 57 months in patients having S100A4-positive involved 4 lymph nodes and more. This was found as 74 months in patients having S100A4-negative. In order to see that S100A4 is a useful parameter for prognosis, it was emphasized that the number of lymph nodes excised in each case should be standardised; because the difference between the numbers of lymph nodes excised in each case may give different and wrong results in the effect of S100A4 on prognosis. More patients and time are required for good results. As for our study, we tried to

show the relationship of the number and percentage of metastatic lymph nodes with S100A4 rather than the number of ideal lymph nodes that should be excised. The prognostic role of S100A4 existence has still been discussed in terms of colorectal cancer (37, 42-47). These disaccords may result from subtle diversities such as the number of patients, observation period, stage distribution, tissue fixation methods, antibody use and cut-off for positivity. During the immunohistochemical staining, not only are the tumour cells stained, but also the parenchymal tissue, reactive fibroblast-like cells, nerve cells, lymphocytes and blood vessels are stained at reduced rate. The staining of tumour cells shows the efficiency of staining. Heterogeneous staining pattern of S100A4 may give wrong results due to non-binding of antigen to antibody. These show up due to the mistakes during staining (48).

In the study that we made, in our opinion, it is quite important to determine the expression level of S100A4 correlated with all other parameters including stage. Especially the occurrence together with the lymph node involvement is valuable for showing the aggressivity of tumour. Considering as a prognostic factor, the occurrence of S100A4 expression in early stages may change the treatment approach in the future. Therefore, further studies should be made with more patients.

In our study, S100A4 expression in colon cancer was defined as a parameter that increases tumor progression and aggressivity. The measurable increase in S100A4 expression at advanced stages in metastatic patients was statistically presented.

Nevertheless, the mechanism of S100A4 protein in stimulating metastasis and invasion is still not fully elucidated. Detecting the expression of this protein at early stages may help prevent future recurrences and metastases, and may even lead to personalized treatment protocols tailored to each patient.

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