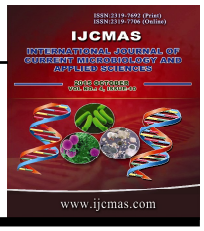
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 138-145

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 138-145**
http://www.ijcmas.com



Original Research Article

Using of S100A9 as a Novel Biomarker for Early Detection of Colorectal Cancer, A Stool Based Study in Iraq

Haider Sabah Kadhim[1], Ahmed A Hussein[2], Dalya B. Hanna[3*], Zahraa Q Ali[2] and Riaydh Z Asmer[5]

[1]College of Medicine/Al-Nahrain University, Iraq
[2]College of Medicine/Baghdad University, Iraq
[3]College of Pharmacy/ University of Mustansiriyah, Iraq
[4]Gastroenterology & Hepatology Hospital/ Baghdad, Iraq
**Corresponding author*

Keywords

Novel biomarker, Colorectal cancer, Histo-pathological diagnosis

A B S T R A C T

Until now, the precise etiology of colorectal cancer (CRC) is not yet known. However, it is widely agreed that CRC develops slowly via a progressive accumulation of genetic mutations and involves inactivation of a variety of tumor-suppressor and DNA – repair genes, with simultaneous activation of certain oncogenes due to long possible list of environmental factors. A total of 60 patients (22 were males and 38 were females) were involved in this study, they were divided according to histopathological diagnosis to three groups, colorectal cancer group, polyp group, and normal colonoscopy group. Samples from patients were selected between May 2012 and August 2014. From each patient enrolled in this study, 2–4 mucosal punch biopsies were taken for histopathology and immunohistochemistry and stool samples were taken and kept in RPMI (Rosewell Park Memorial Institute) medium, to be used in flow cytometry. Statistical analysis of the results showed that there was a significant difference in the positivity rate of S100A9 in histopathology sections and in the stool specimens of the three studied groups. The selected marker, S100A9 in histopathology specimens and in stool samples qualified as significant predictors or risk factors for having malignant CRC compared to healthy controls. While there was no significant difference regarding S100A9 expression as a risk factor between malignant tissues and non neoplastic polyp. And S100A9 in stool samples qualified as significant predictors or risk factors for having malignant CRC compared to healthy controls and non neoplastic polyp. In this study S100A9 showed to be a significant predictor for having CRC in both tissue and stool.

Introduction

Colorectal cancer (CRC) is considered one of the commonest malignant epithelial tumor of the colon or rectum (Imamura *et al.*, 2014) Colorectal cancer initiates in the epithelial cells lining the colon and rectum. The high rate replication of the epithelial cells of the human colon with 10^{10} epithelial cells being replaced every day is thought to

contribute to the vulnerability of colon and rectal epithelium to mutation and consequent carcinogenesis (Komarova, 2005), and many more patients annually suffer morbidity from curative colon cancer surgery and chemotherapy (Win *et al.,* 2013). Both environmental and genetic factors play the main roles in its etiology (Qiu *et al.,* 2012). It is worldwide agreed that accumulation of genetic mutations and epimutations alteration in colonic mucosal cells ultimately leads to cell proliferation and metastasis (Robbins, 2012; Edwards *et al.,* 2012).

There is increasing evidence that altered expression of S100 family members is seen in many cancers including breast, lung, bladder, kidney, thyroid, gastric, prostate and oral cancers. S100 proteins are commonly up-regulated in tumors and this is often associated with tumor progression. In contrast S100A9 also known as S100 calcium-binding protein A9 and S100A2, S100A11 have been documented as tumor suppressors in some cancers but as tumor promoters in others (Hibi *et al.,* 2011).

This demonstrates the complexity of the family and variability of their functions. Although the precise roles of these proteins in cancer is still to be discovered many of the family are associated with promoting metastases through interactions with matrix metalloproteinases or by acting as chemoattractants. There is also evidence that some members can regulate transcription factors such as p53 (Prix *et al.,* 2002; Khoshbaten *et al.,* 2014).

Patients and methods

Patients were selected from those attending the Endoscopic Unit at "Gastroenterology and Hepatology Teaching Hospital/ Baghdad Medical City", Oncology Teaching Hospital/ Baghdad Medical City- Health Directorate, and from the Endoscopic Unit of Baghdad Medical City.

A total of 60 patients (22 were males and 38 were females) were involved in this study (Table 1), they were divided according to histopathological diagnosis to three groups, colorectal cancer group, polyp group, and normal colonoscopy group (Table 2). Samples from patients were selected between May 2012 and August 2014.

From each patient, 2–4 mucosal punch biopsies were taken via sterile standard biopsy forceps and fixed immediately with 10% buffered neutral formalin for preparation of paraffin embedded tissue blocks for histopathology and immunohistochemistry, and stool samples were taken prior to start the preparation for the procedure, or the day the patients would come to take the histopathological report.

Patients also advised to keep the samples frozen. One of the containers was containing RPMI (Rosewell Park Memorial Institute) medium, to be used in flow cytometry.

Histopathological and immuno-histochemical study

Two sections of 6 micrometers thickness were taken from each paraffin embedded tissue block. First sections were put on ordinary slide for Hematoxylin and Eosin (H&E) staining to confirm diagnosis and the second sections were put on the charged slide for immunohistochemistry (IHC) using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436/ Abcam-UK) and Anti-S100A9 antibody [EPR3556] ab92468 to detect the expression of S100A9 in colonic tissue samples.

Human tonsil lysate and peripheral blood leukocyte cell lysate IHC-P: Human spleen tissue was used as a positive control.

DNA extraction and purification from stool samples

Stool samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions.

This obstacle was overcome by using (QIAamp® DNA purification from stool samples)* which is designated for rapid purification of total DNA from stool, and it was suitable for both fresh and frozen samples.

The fast and easy procedures comprised the following steps:

- Lysis of stool samples in Buffer ASL.
- Adsorption of impurities to inhibit EX matrix.
- Purification of DNA on QIAamp Mini spin columns.

Determination of Concentration, Yield, and Purity of DNA*:

DNA yielded were determined from the concentration of the DNA eluted, and was measured by absorbance at 260 nm, by using of Nanodrop. Purity was determined by calculating the ratio of absorbance at 260 nm to the absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7- 1.9.

Flow cytometry for S100A

S100A9 was present in the form of a secreted protein. The detection of any secreted proteins is difficult as the protein will be released from the cell before detection, or may degrade rapidly. Hence, it recommended using Brefaldin A (or other compounds) as a Golgi-Block, where cells were incubated with Brefaldin A which prevents proteins being released from the Golgi. Thereby any cells would expressing the protein can then be detected.

We used Brefeldin A [Brefeldin A Solution (1,000X) ab193369] to this purpose.

Primary antibody for S100A9 used for Flow cytometry was from abcam with cat. No. [ab112228]. Anti-S100A9 antibody [CF-557] was used and the human peripheral blood leukocytes were used as positive control.

Results and Discussion

Difference in positivity rate of tested genes between study groups:

Histopathology sections

The difference was statistically significant in the positivity rate of S100A9 in histopathology sections of the three studied groups (malignant CRC cases, non-neoplastic polyps, and negative –apparently healthy controls), (Table 3), (Figure 1). And the results showed that there was no significant difference in the expression of S100A9 in histopathological section between studied groups (Table 4).

Stool specimens

The positivity rate of S100A9 selected gene was assessed in stool specimens in the three studied groups (malignant CRC cases, non-neoplastic polyps, and negative –apparently healthy controls). The result showed that the difference was statistically significant, (Table 3).

Genes as risk factors for malignancy compared to healthy controls

Histopathology sections

The selected marker, S100A9 in histopathology specimens qualified as significant predictors or risk factors for

having malignant CRC compared to healthy controls. S100A9 remained statistically significant after adjusting for the inflated level of significance attributed to multiple (repeated) significance testing on the same group. The risk of having CRC is increased by 37.1 times in the presence of S100A9. This gene is responsible for around 50% of etiologic fraction for having malignancy (compared to healthy controls) (Table 4).

Stool specimens

The selected marker, S100A9 gene in stool specimens qualified as significant predictors or risk factors for having CRC compared to healthy controls.

This risky gene remained statistically significant even after adjusting for the inflated level of significance attributed to multiple (repeated) significance testing on the same group.

The risk of having CRC is increased by 37.1 times in the presence of S100A9 genes respectively. S100A9 gene was responsible for a high proportion of the total risk for having malignancy (compared to healthy controls), ranging between 58 to 74% of etiologic fraction (Table 4).

Genes as risk factors for malignancy compared to non-neoplastic polyp

Histopathology sections

Although a positive S100A9 gene in histopathology specimen increases the risk of having CRC by 4.5 times compared to non-neoplastic polyp, the calculated risk estimate failed short of statistical significance, possibly because of small sample size. This gene was responsible for 46.7% of total risk for having malignancy as opposed to non-malignant polyp.

Stool specimens

S100A9 tested gene in stool specimens qualified as significant predictors or risk factors for having malignant CRC compared to non-neoplastic polyp. This gene remained statistically significant after adjusting for the inflated level of significance attributed to multiple (repeated) significance testing on the same group. The risk of having CRC is increased by 25.2 times in the presence of S100A9, which is responsible for around 60% of etiologic fraction for having malignancy as opposed to non-neoplastic polyp.

Gene expression in tumorous tissue versus stool specimens among cases with colorectal cancer

The expression of S100A9 in biopsy material obtained from tumorous tissue was compared to stool specimens obtained from cases with colonic cancer. Each histologic specimen was paired with a stool sample obtained from the same patient.

S100A9 showed an exactly equivocal tendency to be expressed in histologic specimens and stool samples. This tendency was however not significant statistically, as detailed in table 6.

Colorectal cancer is one of the most common malignancies affecting both sexes and a common cause of mortality worldwide. The present findings, along with earlier studies, show that S100A8/A9 function at multiple stages in disease progression. S100A8/A9, their receptors and signaling pathways therefore provide important targets for development of pharmacological interventions and for the identification of early-stage disease biomarkers (Mie Ichikawae *et al.,* 2011).

The inconsistent association of S100A expression with clinicopathological features and patient prognosis among cancers may be caused by the complexity of the cancer microenvironment.

In the current study, unexpected results regarding S100A9 were obtained. When comparing the difference in expression rate among the study groups (CRC, non-neoplastic polyp, normal colonic tissue), this biomarker showed the same (60%) expression in CRC of tissue and stool samples and, (25%) in contrast to (0%) in non- neoplastic polyp samples in both tissue and stool samples respectively. In colorectal carcinoma, tumoral tissues infiltrate with various immune/inflammatory cells along their invasive margins, and the increased S100A8/A9 expression in these immune cells infiltrating the tumor has been demonstrated by certain studies. In addition, several studies have suggested that increased S100A8/A9 expression in colorectal carcinoma may play a role in tumor progression and may be associated with metastasis and histological grade.

Although a few studies imply a possible relationship with tumor progression and metastasis, there is no clear correlation between increased expression of S100A8/A9 in colorectal cancer and clinicopathological parameters such as tumor progression, tumor stage, lymph node metastases, or distant metastases (Cumhur İbrahim Başsorgun *et al.*, 2014).

On the other hand, when applying other statistical analyses to this biomarker, it showed to be a significant predictor for having CRC in both tissue and stool. When comparing CRC to normal and more, it had 37.1 times to have the tendency to be expressed in CRC on the count of normal tissue samples, which was the same result when comparing the same two groups but using stool samples, making it a significant predictor for CRC.

Moreover, even after adjusted for the effect of multiple comparisons, S100A9 showed 25.2 times significantly higher in CRC more than non- neoplastic polyp when using stool samples. Still, the S100A9 showed significant higher tendency to be expressed by 12 times (60%) more in tumor tissue rather than in the normal adjacent one of the same patient. More interestingly, it showed no association to the family history, sex, or age.

Although it is a fact that this is the first local study dealing with S100A9, and more, in its presence in stool of CRC patients, but, it is also a fact that we lost the privilege in using the local related data to explain the results.

Table.1 Represents sex allotment

Sex	CRC (%)	Polyp (%)	Normal (%)	Total
Female	28	5	5	38
Male	12	3	7	22
Total	40	8	12	60

Table.2 Patient's categorization

Group	Endoscopy finding	Number of patients
I	Tumor * (CRC)	40
II	Polyp	12
III	Normal colonoscopy	8
Total		60

*CRC- Colorectal Cancer/ proved according to histopathological report

Table.3 The difference in histopathology (affected tissue) and in stool specimens positivity rate of S100A9 between the 3 study groups

Positive gene-stool	Studied groups						P(Chisquare test)
	(apparently healthy) controls (n=12)		polyp controls (non-neoplastic) (n=8)		Malignant cases (CRC) (n=40)		
	N	%	N	%	N	%	
S100A9-affected tissue	**0**	**0.0**	**2**	**25.0**	**24**	**60.0**	**<0.001**
S100A9-stool	**0**	**0.0**	**0**	**0.0**	**24**	**60.0**	

Table.4 The histopathology (affected tissue) specimens' positivity rate of S100A9 by gender among cases with malignant CRC

Positive gene-affected tissue	Gender				P (Chi-square)
	Female (n=28)		Male (n=12)		
	N	%	N	%	
S100A9	**17**	**60.7**	**7**	**58.3**	**1[NS]**

Table.5 The risk of having CRC compared to healthy controls for S100A9 identified in histopathology (affected tissue) and stool specimens

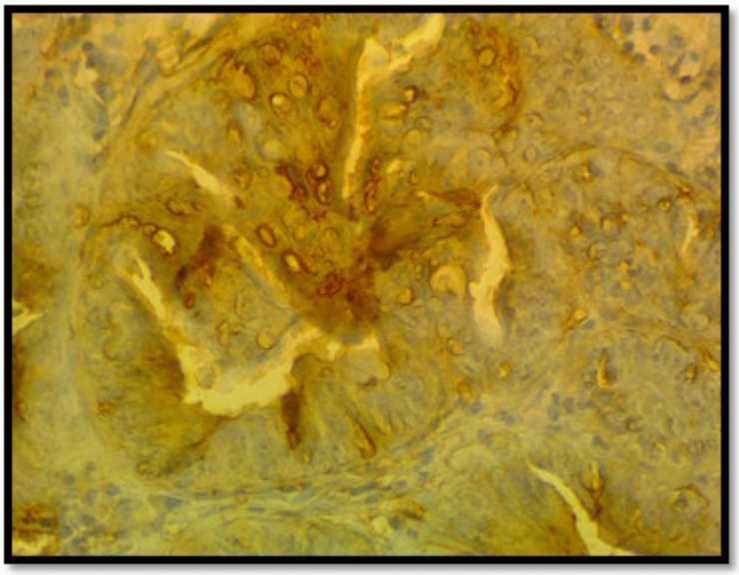
Positive gene	Negative (apparently healthy) controls (n=12)		Malignant cases (CRC) (n=40		OR	Inverse OR	EF	PF	Fisher's exact	Adjusted P
	N	%	N	%						
S100A9-affected tissue	**0**	**0.0**	**24**	**60.0**	**37.1**	**	**0.584**	**	**<0.001**	**0.002**
S100A9-stool	**0**	**0.0**	**24**	**60.0**	**37.1**	**	**0.584**	**	**<0.001**	**0.002**

Positive gene	Negative (apparently healthy) controls (n=12)		Malignant cases (CRC) (n=40		OR	Inverse OR	EF	PF	Fisher's exact	Adjusted P
	N	%	N	%						
S100A9-affected tissue	**2**	**25.0**	**24**	**60.0**	**4.5**	**	**0.467**	**	**0.064**	
S100A9-stool	**0**	**0.0**	**24**	**60.0**	**25.2**	**	**0.576**	**	**0.002**	**0.021**

Table.6 Comparing the performance of selected tests in histopathologic specimens for affected tissue (tumor) compared to stool exam among cases with malignancy (CRC)

S100A9 –affected tissue(pathological)	Same marker-stool exam					
	Negative		Positive		Total	
	N	%	N	%	N	%
Negative	14	87.5	2	12.5	16	100
Positive	2	8.3	22	91.7	24	100
Total	16	40	24	60	40	100
There is a equal tendency for examined stool to be positive compared to affected tissue Positivity rate for affected (tumor) tissue= 60% Positivity rate for stool=60% P (McNemar) =1 [NS] (not significant)						

Figure.1 Diffused expression of S100A9 mAb in Colon cancer tissue.
Formalin fixed, Paraffin embedded



The tumor microenvironment plays an important role in modulating tumor progression. It has been suggested that calcium ions play a significant role in the development of colorectal carcinoma through a direct impact on proliferation and differentiation via calcium receptors. For this reason, increased expression of calcium-binding proteins S100A8 and A9, members of the S-100 protein family, in the peritumoral and intratumoral spaces in colorectal carcinoma becomes important for tumor progression. Therefore, the analyses conducted on the expression of these proteins and tumor behavior suggests that S100A8/A9 can become potential therapeutic targets in cancer treatment (El-Rifai *et al.*, 2002). So far, a limited number of studies have reported correlation between increased expression of S100A8/A9 and parameters such as tumor differentiation, metastasis, tumor size, and Dukes stage (Cumhur İbrahim Başsorgun *et al.*, 2014). However, further studies with larger sample sizes are warranted to substantiate the results of this study and to better understand the functions of the S100A8/A9 positive immune cells observed in the tumor microenvironment of colorectal cancer in tumorigenesis and tumor progression.

In conclusion, S100A9 was served as a good test to diagnose CRC differentiating it from non- neoplastic polyp using stool samples. And it is the positive gene that served as a good test to diagnose CRC differentiating it apparently healthy controls using stool samples.

Reference

Cumhur İbrahim Başsorgun, Betül Ünal, Nuray Erin, Anıl Özlük, Özlem Ceren Uzun, Gülsüm Özlem Elpek, 2014. S100A8 and S100A9 positive cells in colorectal carcinoma: clinicopathological analysis. *Gastroenterol. Res. Pract.,* 2014: 6.

Edwards, M.S., *et al.* 2012. A systematic review of treatment guidelines for metastatic colorectal cancer. *Colorectal Dis.*, 14(2): e31–47.

El-Rifai, C.. Moskaluk, M.. Abdrabbo, K., *et al.* 2002. Gastric cancers overexpress S100A calcium-binding proteins. *Cancer Res.*, 62(23): 6823–6826.

Hibi, K., *et al.* 2011. Detection of TFPI2 methylation in the serum of gastric cancer patients. *Anticancer Res.*, 31(11): 3835–3838.

Imamura, Y., *et al.* 2014. Analyses of clinicopathological, molecular, and prognostic associations of KRAS codon 61 and codon 146 mutations in colorectal cancer: cohort study and literature review. *Mol. Cancer,* 13: 135.

Khoshbaten, M., *et al.* 2014. Diagnostic value of fecal calprotectin as a screening biomarker for gastrointestinal malignancies. *Asian Pac. J. Cancer Prev.*, 15(4): 1667–1670.

Komarova, N.L. 2005. Cancer, aging and the optimal tissue design. *Semin Cancer Biol.*, 15: 494–505.

Mie Ichikawa, Roy Williams, Ling Wang, Thomas Vogl, Geetha Srikrishna, 2011. S100A8/A9 activate key genes and pathways in colon tumor progression. *Mol. Cancer Res.*, 9(2): 133–148.

Prix, L., *et al.* 2002. Diagnostic biochip array for fast and sensitive detection of K-ras mutations in stool. *Clin. Chem.*, 48(3): 428–435.

Qiu, R., *et al.* 2012. Large conformational changes in MutS during DNA scanning, mismatch recognition and repair signalling. *EMBO J.*, 31(11): 2528–2540.

Robbins, 2012. Cell injury, cell death, and adaptations. In: Kumar, V., Abbas, A.K., Aster, J.C. (Eds.), Robbins basic athology. Elsevier, Philadelphia. Pp. 1–28.

Win, A.K., *et al.* 2013. Criteria and prediction models for mismatch repair gene mutations: a review. *J. Med. Genetics,* 50(12): 785–793.