Original Research Article

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

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ABSTRACT

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.

Keywords
Novel biomarker, Colorectal cancer, Histo-pathological diagnosis

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 immunohistochemical 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 S100A9**

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
hav[ing 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

<table>
<thead>
<tr>
<th>Sex</th>
<th>CRC (%)</th>
<th>Polyp (%)</th>
<th>Normal (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>28</td>
<td>5</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>3</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>8</td>
<td>12</td>
<td>60</td>
</tr>
</tbody>
</table>
**Table 2** Patient's categorization

<table>
<thead>
<tr>
<th>Group</th>
<th>Endoscopy finding</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Tumor <em>(CRC)</em></td>
<td>40</td>
</tr>
<tr>
<td>II</td>
<td>Polyp</td>
<td>12</td>
</tr>
<tr>
<td>III</td>
<td>Normal colonoscopy</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Positive gene-stool</th>
<th>Studied groups</th>
<th>Malignant cases (CRC) (n=40)</th>
<th>P(Chisquare test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(apparently healthy controls) (n=12)</td>
<td>polyp controls (non-neoplastic) (n=8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>S100A9-affected tissue</td>
<td>0</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td>S100A9-stool</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Positive gene-affected tissue</th>
<th>Female (n=28)</th>
<th>Male (n=12)</th>
<th>P (Chisquare)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>S100A9</td>
<td>17</td>
<td>60.7</td>
<td>7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Positive gene</th>
<th>Negative (apparently healthy controls) (n=12)</th>
<th>Malignant cases (CRC) (n=40)</th>
<th>OR</th>
<th>Inverse OR</th>
<th>EF</th>
<th>PF</th>
<th>Fisher's exact</th>
<th>Adjusted P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100A9-affected tissue</td>
<td>0</td>
<td>0.0</td>
<td>24</td>
<td>60.0</td>
<td>37.1</td>
<td>**</td>
<td>0.584</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S100A9-stool</td>
<td>0</td>
<td>0.0</td>
<td>24</td>
<td>60.0</td>
<td>37.1</td>
<td>**</td>
<td>0.584</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 6 Comparing the performance of selected tests in histopathologic specimens for affected tissue (tumor) compared to stool exam among cases with malignancy (CRC)

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

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 Başşorgun 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


