Author's response to reviews

Title: The calcium-binding protein S100P in normal and malignant human tissues

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Author's response to reviews: see over
Dear Editor,

We were grateful for the constructive critiques our manuscript (The calcium-binding protein S100P in normal and malignant human tissues) received and feel that we were able to respond to questions or suggestions with changes that greatly improved the manuscript. I hope that you will find the revised version of the manuscript acceptable for publication in the BMC Clinical Pathology.

Attached is a listing of the detailed responses to each of the reviewers’ comments.

Sincerely yours,

Professor Seppo Parkkila, M.D., Ph.D.
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**Response to the comments from the referee 1:**

The reviewer pointed out that it was not clear how many tissue samples were used for each column in Figure 1 and variations should be indicated with error bars.

1. The cDNA samples from different human tissues are commercially available. Based on the text in datasheet and information obtained from the company the cDNAs were prepared from poly(A) RNAs isolated from pooled organs and cell fractions. Since there were always several patient samples (n=4-550) we believe that the results obtained are representative enough. We cannot provide error bars because of sample pooling. We have added the following information (Page 5, line 16):

   “The numbers of pooled tissue specimens for each RNA sample were as follows: placenta (n=7), spleen (n=11), thymus (n=18), prostate (n=32), testis (n=45), ovary (n=5), leukocyte (n=550), ascending colon (n=5), descending colon (n=7), transverse colon (n=19), duodenum (n=30), ileocecum (n=19), ileum (n=8), jejunum (n=6), rectum (n=6), cecum (n=29), stomach (n=7), esophagus (n=39), mononuclear cells (n=12), resting CD8+ cells (n=20), resting CD4+ cells (n=11), resting CD14+ cells (n=36), resting CD19+ cells (pooled from Caucasian blood donors, number not provided), activated CD19+ cells (n=4), activated mononuclear cells (n=4), activated CD4+ cells (n=6) and activated CD8+ cells (n=8).”

2. Like all the other reviewers also this referee requested more detailed characterization of the newly generated monoclonal S100P antibody. We have performed several
experiments which demonstrated that the antibody is specific for S100P protein and it recognizes a different epitope than the commercially available antibody. We have added the following information:

(Page 7, line 8):

**Immunoprecipitation and Western blotting**

Cells grown in confluent monolayer were rinsed twice with cold PBS and solubilised in ice-cold RIPA buffer (1% Triton X-100 and 1% deoxycholate in PBS) containing the commercial COMPLETE cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. The extracts were collected, cleared by centrifugation at 15 000 rpm for 10 min at 4°C and stored at -80°C. Protein concentrations of extracts were quantified using the BCA protein assay reagent (Pierce, Rockford, IL).

The extracts were incubated overnight with the MAb 18-9 and in parallel with the control MAb. The immunocomplexes were collected on 25 µl 50% suspension of Protein-G Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C and removed from the extract by centrifugation at 15 000 rpm for 2 min.

The extracts were resolved in 12% SDS-PAGE and transferred to PVDF membrane (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). After blocking in 5% non-fat dry milk with 0.2% Nonidet P40 in PBS, the membrane was probed with MAbs, washed and treated with secondary anti-mouse HRP-conjugated swine antibody diluted 1/7500 (Sevapharma, Prague, Czech Republic). The protein bands were visualized by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech).

**Competitive antibody-binding ELISA**

Extract from HeLa cells was adsorbed on microplate wells at a concentration corresponding to 50% of maximal binding of labelled 18-9 MAb. Coated plates were washed and saturated with 10% fetal calf serum (FCS) in PBS. Serial tenfold dilutions of purified 18-9 and control MAbs in 30 µl and a constant amount of biotinylated 18-9 MAb in 30 µl were added and incubated overnight at 4°C. The plates were washed and peroxidase-labelled streptavidin (Pierce) was used as a detector.

(Page 10, line 22):

“For this purpose, we used a newly generated anti-S100P monoclonal antibody 18-9 produced against a recombinant fusion GST-S100P antigen. First, we verified the specificity of the MAb in comparison with the commercially available antibody designated here as a “control MAb” (Fig. 2). In Western blotting, both antibodies recognized an 11 kDa polypeptide in the extract from placenta (Fig. 2A). However, only the 18-9 MAb reacted well with the purified GST-S100P protein, possibly because the control antibody was raised against a different form of the antigen (Fig. 2A). Removal of S100P from the extract of HeLa cells by Protein G-mediated immunoprecipitation with the control MAb resulted in considerably reduced S100P signal visualized by 18-9 MAb and vice versa (Fig. 2B), demonstrating that both antibodies recognize the same antigen and neither of them has a cross-reactivity with S100A proteins expressed in HeLa cells [17]. In addition, 18-9 MAb gave the same pattern of reactivity in HeLa (S100P positive),
HeLa-R (S100P-negative variant of HeLa cells) and HeLa-R cells transfected with S100P cDNA (Fig. 2C) when compared to the control MAb, see [9]. Noteworthy, a competitive ELISA using the biotinylated 18-9 MAb revealed that these two antibodies bind to different antigenic sites on S100P molecule (Fig. 2D). The specificity of the 18-9 MAb was also evaluated by immunohistochemistry in comparison with the control MAb in parallel sections of the human stomach antrum (Fig. 3). Both antibodies revealed strong positive immunoreactions in the surface epithelial cells. The control MAb showed slightly stronger reactivity in the glands and lamina propria. All these results clearly demonstrated that the 18-9 MAb is specific for S100P protein and approved its use for the immunohistochemical analysis of S100P expression in human tissues (Figs 4 and 5) and carcinomas including breast (Fig. 6A,B), gastric (Fig. 6C), pancreatic (Fig. 6D), ovarian (Fig. 6E,F), prostate, and colon (Fig. 7) tumors.”

In addition, we have added new figures 2 and 3 and the corresponding figure legends.

3. The reviewer pointed out that the discrepancy between the protein and mRNA levels in esophagus could be interesting, but needs to be confirmed on larger numbers of specimens.
We performed additional immunostainings on six esophagus specimens. The results showed that there is some variation in the staining reactivity between different samples. We have changed the panel F in Figure 4. The new panel shows a slightly stronger immunostaining than the previous version of the figure, although the immunoreactions remained clearly weaker in all esophagus specimens than in the stomach. We also extended this study to include fifteen specimens of oral mucosa (containing similar stratified squamous epithelium). The immunostaining reactions in the oral epithelium were also weak or moderate. Since this finding did not change the main conclusion about the staining reactivity in the esophagus, we did not include this additional data in the text. We slightly modified the following sentence:
(Page 14, line 24): “…the esophagus which repeatedly showed high mRNA signals and relatively weak or moderate immunohistochemical staining.”

4. The reviewer pointed out that the text was somewhat vague in places which he mentioned. We have modified the text as follows:
(Page 12, line 20): “These figures indicate that the staining reactivity was higher in most tumors (54%) compared to the adjacent benign tissue.”
Unfortunately, we were not able to provide more data on a large series of 1500 breast cancers at this stage. Our pathologist indicated that this large series of breast tumor specimens has been collected and characterized by a large consortium of researchers. More detailed information will be provided in another study using variety of other tumor
markers. We agree with the reviewer that the sentence mentioning this larger set of tumors was not clear and therefore we have eliminated it from the revised version.

5. The reviewer mentioned that it is difficult to see why the widespread occurrence of S100P is not a bar for therapeutic applications.

We agree with the reviewer that the expression of S100P in several normal tissues raises concerns whether it can serve as a therapeutic target. However, there are other well-known examples where a potential or even a clinically used therapeutic target is expressed in normal tissues in addition to tumor cells. These include for example ErbB-2 (Pancreatology 2005;5:44-58 ; Recent Prog Horm Res 2004;59:1-12 ; J Leukoc Biol 2003 Oct;74:593-601) and VEGF (Microvasc Res 2007 Apr 6; [Epub ahead of print]). Certainly, it will be a challenge to find a good target which is solely expressed in tumors. In this respect, S100P can be still considered a potential target protein.

Since the second reviewer also pointed out that S100P could still represent a useful diagnostic marker, we have slightly modified the text as follows:

(Page 2, line 16): “Based on our observations, S100P is widely expressed in both normal and malignant tissues. The high expression in some tumors suggests that it may represent a potential target molecule for future diagnostic and therapeutic applications.”

(Page 15, line 17): “It clearly shows ectopic expression in some cancers. Based on the high expression in certain tumors, S100P could represent a potential target for novel diagnostic and therapeutic applications.”

6. The reviewer pointed out that the last paragraph of the discussion should be more focused and suggested that speculation related to interleukin should be removed. We deleted the last paragraph and wrote another one which is more related to the discussion on diagnostic and therapeutic aspects of S100P. The new text reads: “Structural studies have shown that S100P protein exists as a dimer and the S100P homodimer is probably more stable than those of other S100 proteins [25]. High stability is a prerequisite for a good biomarker. In this respect, S100P gene or protein expression has already been proved to correlate with patient survival in lung [7, 26] and breast cancer [15], and it has been proposed as an early developmental marker of pancreatic carcinogenesis [19]. Our present results using a newly generated monoclonal S100P antibody confirmed the expression of S100P protein in several tumor categories. It is noteworthy, however, that S100P is not restricted to neoplastic cells, but is also detectable in various normal cell types. This fact has to be carefully considered when planning novel diagnostic and therapeutic applications based on S100P expression.”

Minor essential revisions:

1. The reviewer noted that the figure legend 2 (presently figure 4) contained some mislabelling. It is now corrected.

2. The reviewer suggested that the title should read “The” calcium binding protein… We have corrected the title as suggested.

3. The reviewer requested a reference to the statement at the beginning of the discussion. We have added there two references (18,19).
Response to the comments from the referee 2:

1. Like all the other reviewers also this referee requested more detailed characterization of the newly generated monoclonal S100P antibody. We have performed several experiments which demonstrated that the antibody is specific for S100P protein and it recognizes a different epitope than the commercially available antibody. We have added the following information:

   (Page 7, line 8):
   "Immunoprecipitation and Western blotting
   Cells grown in confluent monolayer were rinsed twice with cold PBS and solubilised in ice-cold RIPA buffer (1% Triton X-100 and 1% deoxycholate in PBS) containing the commercial COMPLETE cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. The extracts were collected, cleared by centrifugation at 15 000 rpm for 10 min at 4°C and stored at -80°C. Protein concentrations of extracts were quantified using the BCA protein assay reagent (Pierce, Rockford, IL).

   The extracts were incubated overnight with the MAb 18-9 and in parallel with the control MAb. The immunocomplexes were collected on 25 µl 50% suspension of Protein-G Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C and removed from the extract by centrifugation at 15 000 rpm for 2 min.

   The extracts were resolved in 12% SDS-PAGE and transferred to PVDF membrane (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). After blocking in 5% non-fat dry milk with 0.2% Nonidet P40 in PBS, the membrane was probed with MAb, washed and treated with secondary anti-mouse HRP-conjugated swine antibody diluted 1/7500 (Sevapharma, Prague, Czech Republic). The protein bands were visualized by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech).

   Competitive antibody-binding ELISA
   Extract from HeLa cells was adsorbed on microplate wells at a concentration corresponding to 50% of maximal binding of labelled 18-9 MAb. Coated plates were washed and saturated with 10% fetal calf serum (FCS) in PBS. Serial tenfold dilutions of purified 18-9 and control MAbs in 30 µl and a constant amount of biotinylated 18-9 MAb in 30 µl were added and incubated overnight at 4°C. The plates were washed and peroxidase-labelled streptavidin (Pierce) was used as a detector."

   (Page 10, line 22):
   "For this purpose, we used a newly generated anti-S100P monoclonal antibody 18-9 produced against a recombinant fusion GST-S100P antigen. First, we verified the specificity of the MAb in comparison with the commercially available antibody designated here as a “control MAb” (Fig. 2). In Western blotting, both antibodies recognized an 11 kDa polypeptide in the extract from placenta (Fig. 2A). However, only the 18-9 MAb reacted well with the purified GST-S100P protein, possibly because the control antibody was raised against a different form of the antigen (Fig. 2A). Removal of S100P from the extract of HeLa cells by Protein G-mediated immunoprecipitation with
the control MAb resulted in considerably reduced S100P signal visualized by 18-9 MAb and vice versa (Fig. 2B), demonstrating that both antibodies recognize the same antigen and neither of them has a cross-reactivity with S100A proteins expressed in HeLa cells [17]. In addition, 18-9 MAb gave the same pattern of reactivity in HeLa (S100P positive), HeLa-R (S100P-negative variant of HeLa cells) and HeLa-R cells transfected with S100P cDNA (Fig. 2C) when compared to the control MAb, see [9]. Noteworthy, a competitive ELISA using the biotinylated 18-9 MAb revealed that these two antibodies bind to different antigenic sites on S100P molecule (Fig. 2D).

The specificity of the 18-9 MAb was also evaluated by immunohistochemistry in comparison with the control MAb in parallel sections of the human stomach antrum (Fig. 3). Both antibodies revealed strong positive immunoreactions in the surface epithelial cells. The control MAb showed slightly stronger reactivity in the glands and lamina propria. All these results clearly demonstrated that the 18-9 MAb is specific for S100P protein and approved its use for the immunohistochemical analysis of S100P expression in human tissues (Figs 4 and 5) and carcinomas including breast (Fig. 6A,B), gastric (Fig. 6C), pancreatic (Fig. 6D), ovarian (Fig. 6E,F), prostate, and colon (Fig. 7) tumors.”

(Page 14, line 7):
“In the present study, we used a newly generated anti-S100P monoclonal antibody 18-9 for immunohistochemical analysis of S100P expression in a series of normal and tumor human tissues. Comparative analysis of the new 18-9 MAb and the commercial control MAb confirmed that these two MAbs are both specific for S100P protein, but bind to different epitopes.”

In addition, we have added new figures 2 and 3 and the corresponding figure legends.

2. Like the reviewer #1 also this reviewer pointed out that S100P protein could still be a reliable marker of malignancy and the widespread expression might indicate limitations as a therapeutic target.

We agree with the reviewer that the expression of S100P in several normal tissues raises concerns whether it can serve as a therapeutic target. However, there are other well-known examples where a therapeutic target is expressed in normal tissues in addition to tumor cells. These include for example ErbB-2 (Pancreatology 2005;5:44-58; Recent Prog Horm Res 2004;59:1-12; J Leukoc Biol 2003 Oct;74:593-601) and VEGF (Microvasc Res 2007 Apr 6; [Epub ahead of print]). Certainly, it will be a challenge to find a good target which is solely expressed in tumors. In this respect, S100P can be still considered a potential target protein.

Since the first reviewer also pointed out that S100P could still represent a useful diagnostic marker, we have slightly modified the text as follows:
(Page 2, line 16): “Based on our observations, S100P is widely expressed in both normal and malignant tissues. The high expression in some tumors suggests that it may represent a potential target molecule for future diagnostic and therapeutic applications.”
(Page 15, line 17): “It clearly shows ectopic expression in some cancers. Based on the high expression in certain tumors, S100P could represent a potential target for novel diagnostic and therapeutic applications.”
Minor essential revisions:
1. The reviewer requested information about the primers whether they spanned an exon-exon junction. We have added the following information (Page 5, line 6): “The primers were located in two different exons separated by a 2822 bp-long intron.” In addition we confirmed by sequencing that the PCR-product represented S100P sequence.
2. The composition of the Wash buffer is now provided on Page 8, line 20.
3. The reviewer noted that the figure legend 2 (presently figure 4) contained some mislabelling. It is now corrected.

Discretionary revisions:
1. The reviewer pointed out that the previous version of the manuscript contained detailed discussion about S100P in leukocytes, which he did not consider important in this paper. This part of the discussion (the last paragraph) was deleted and we wrote another one which is more related to the discussion on diagnostic and therapeutic aspects of S100P. The new text reads: “Structural studies have shown that S100P protein exists as a dimer and the S100P homodimer is probably more stable than those of other S100 proteins [25]. High stability is a prerequisite for a good biomarker. In this respect, S100P gene or protein expression has already been proved to correlate with patient survival in lung [7, 26] and breast cancer [15], and it has been proposed as an early developmental marker of pancreatic carcinogenesis [19]. Our present results using a newly generated monoclonal S100P antibody confirmed the expression of S100P protein in several tumor categories. It is noteworthy, however, that S100P is not restricted to neoplastic cells, but is also detectable in various normal cell types. This fact has to be carefully considered when planning novel diagnostic and therapeutic applications based on S100P expression.”
Response to the comments from the referee 3:

In the general comments, the reviewer pointed out that the rationale for the study was not clearly defined. We think that the last paragraph of the Introduction provides the rationale clearly enough: “In the present study, we describe a novel monoclonal antibody for S100P protein designated 18-9 and evaluate S100P expression in normal and neoplastic human tissues by immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (RT-PCR). This data could provide valuable information of where S100P is expressed under normal and pathological conditions, and whether it could serve as a tissue- or tumor-specific biomarker.” The availability of different human samples, of course, very much dictated what we were able to analyze. Studies of different leukocyte fractions was justified, because S100P has been recently linked to various leukocyte disorders (e.g. Clin Cancer Res 2006;12:4812-21 ; Eur J Pediatr. 2005;164:427-31 ; Diagn Pathol 2007;2:9).

1. The reviewer suggested that the specificity of the primers should be tested by sequencing of the PCR-product. We confirmed it as suggested and the corresponding text has been added on (Page 10, line 4): “The PCR product from placenta was sequenced in order to confirm its identity and to exclude any unspecific binding of primers (data not shown).”

2. Like all the other reviewers also this referee requested more detailed characterization of the newly generated monoclonal S100P antibody. We have performed several experiments which demonstrated that the antibody is specific for S100P protein and it recognizes a different epitope than the commercially available antibody. We have added the following information:

(Page 7, line 8):

**“Immunoprecipitation and Western blotting”**

Cells grown in confluent monolayer were rinsed twice with cold PBS and solubilised in ice-cold RIPA buffer (1% Triton X-100 and 1% deoxycholate in PBS) containing the commercial COMPLETE cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. The extracts were collected, cleared by centrifugation at 15 000 rpm for 10 min at 4°C and stored at -80°C. Protein concentrations of extracts were quantified using the BCA protein assay reagent (Pierce, Rockford, IL).

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For this purpose, we used a newly generated anti-S100P monoclonal antibody 18-9 produced against a recombinant fusion GST-S100P antigen. First, we verified the specificity of the MAb in comparison with the commercially available antibody designated here as a “control MAb” (Fig. 2). In Western blotting, both antibodies recognized an 11 kDa polypeptide in the extract from placenta (Fig. 2A). However, only the 18-9 MAb reacted well with the purified GST-S100P protein, possibly because the control antibody was raised against a different form of the antigen (Fig. 2A). Removal of S100P from the extract of HeLa cells by Protein G-mediated immunoprecipitation with the control MAb resulted in considerably reduced S100P signal visualized by 18-9 MAb and vice versa (Fig. 2B), demonstrating that both antibodies recognize the same antigen and neither of them has a cross-reactivity with S100A proteins expressed in HeLa cells [17]. In addition, 18-9 MAb gave the same pattern of reactivity in HeLa (S100P positive), HeLa-R (S100P-negative variant of HeLa cells) and HeLa-R cells transfected with S100P cDNA (Fig. 2C) when compared to the control MAb, see [9]. Noteworthy, a competitive ELISA using the biotinylated 18-9 MAb revealed that these two antibodies bind to different antigenic sites on S100P molecule (Fig. 2D).

The specificity of the 18-9 MAb was also evaluated by immunohistochemistry in comparison with the control MAb in parallel sections of the human stomach antrum (Fig. 3). Both antibodies revealed strong positive immunoreactions in the surface epithelial cells. The control MAb showed slightly stronger reactivity in the glands and lamina propria. All these results clearly demonstrated that the 18-9 MAb is specific for S100P protein and approved its use for the immunohistochemical analysis of S100P expression in human tissues (Figs 4 and 5) and carcinomas including breast (Fig. 6A,B), gastric (Fig. 6C), pancreatic (Fig. 6D), ovarian (Fig. 6E,F), prostate, and colon (Fig. 7) tumors.

In the present study, we used a newly generated anti-S100P monoclonal antibody 18-9 for immunohistochemical analysis of S100P expression in a series of normal and tumor human tissues. Comparative analysis of the new 18-9 MAb and the commercial control MAb confirmed that these two MAbs are both specific for S100P protein, but bind to different epitopes.

In addition, we have added new figures 2 and 3 and the corresponding figure legends.
3. The reviewer asked whether the immunostaining was evaluated from the most intensely stained areas. The previous version of the text might have given a wrong impression about the scoring system, because we mentioned only the staining intensity. The evaluation covered equally both the intensity and extent of the staining. A better phrase is “the staining reactivity” which we have added to the figure 8.

Discretionary revisions:
1. We have shortened the introduction concerning to S100P regulation by androgen and interleukin as suggested by the reviewer.
2. We agree with the reviewer that a table summarizing immunohistochemical results could be useful. In principle, the same data can be seen in the figures. We can provide the table if necessary, but would like to leave this decision to the editor.