Immunohistochemical assessment of mitochondrial superoxide dismutase (MnSOD) in colorectal premalignant and malignant lesions

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Key words: colorectal cancer, colorectal adenoma, manganese superoxide dismutase, immunohistochemistry, oxidative stress.

Abstract

Introduction: It is generally accepted that mitochondria are a primary source of intracellular reactive oxygen species (ROS). Under physiological circumstances they are permanently formed as by-products of aerobic metabolism in the mitochondria. To counter the harmful effect of ROS, cells possess an antioxidant defence system to detoxify ROS and avert them from accumulation at high concentrations. Mitochondria-located manganese superoxide dismutase (MnSOD, SOD2) successfully converts superoxide to the less reactive hydrogen peroxide (H₂O₂). To the best of our knowledge, there are no available data regarding immunohistochemical expression of MnSOD in colorectal neoplastic tissues. 

Aim: To investigate the immunohistochemical expression status of MnSOD in colorectal premalignant and malignant lesions.

Material and methods: This study was performed on resected specimens obtained from 126 patients who had undergone surgical resection for primary sporadic colorectal cancer, and from 114 patients who had undergone colonoscopy at the Municipal Hospital in Jaworzno (Poland). Paraffin-embedded, 4-μm-thick tissue sections were stained for rabbit polyclonal anti SOD2 antibody obtained from GeneTex (clone TF9-10-H10 from America Diagnostica).

Results: Results of our study demonstrated that the development of colorectal cancer is connected with increased expression of MnSOD both in adenoma and adenocarcinoma stages. Samples of adenocarcinoma with G2 and G3 grade showed significantly higher levels of immunohistochemical expression of this antioxidant enzyme. Moreover, patients with the presence of lymphovascular invasion and higher degree of regional lymph node status have been also characterised by higher levels of MnSOD expression. The samples of adenoma have been characterised by higher levels of MnSOD expression in comparison to normal mucosa as well. Interestingly, there was no significant correlation between expression and histological type of adenoma.

Conclusions: Development of colorectal cancer is connected with increased expression of MnSOD both in adenoma and adenocarcinoma stages.

Introduction

It is generally accepted that mitochondria are a primary source of intracellular reactive oxygen species (ROS). Under physiological circumstances they are permanently formed as by-products of aerobic metabolism in the mitochondria [1]. During mitochondrial respiration, the tricarboxylic acid (TCA) cycle generates reducing components acting as a source for electrons. Electron transfer between mitochondrial electron transport complexes sets up a proton gradient for ATP synthesis [2]. Nevertheless, it has been documented that electrons can flee from the electron transport chain and react with oxygen molecules to form superoxide anions, which is the primary member in a wide array of the ROS family. It is worth mentioning that roughly 1–5% of the total oxygen consumed during respiration process is...
adenomatous polyps, commonly called adenomas. Al-
colorectal cancer arises from the lesions known as
expression of MnSOD in colorectal neoplastic tissues.
are no available data regarding immunohistochemical
igtional tract [9]. To the best of our knowledge, there
dant role in maintenance of homeostasis in the gastro-
mental role in ROS detoxification [8]. Because superoxide primarily emerges from mitochon-
dria, mitochondrial MnSOD is thought to have a fundament-
role in ROS detoxification [8].
As revealed by studies, MnSOD plays a very signifi-
c role in maintenance of homeostasis in the gastro-
testinal tract [9]. To the best of our knowledge, there
are no available data regarding immunohistochemical
expression of MnSOD in colorectal neoplastic tissues.
Colorectal cancer arises from the lesions known as
adenomatous polyps, commonly called adenomas. Al-
though every adenoma has the capacity of malignant
transformation very few adenomas develop into cancer [10]. A survey comparing adenoma prevalence and car-
cinoma incidence demonstrated that the percentage of
transformation is about 0.25% per year [11].

Aim
Therefore, in the present study we investigated the
immunohistochemical expression status of MnSOD in
colorectal premalignant and malignant lesions. The
 correlations with significant clinicopathological vari-
bles and MnSOD expression in colorectal cancer and
adenoma patients were also determined.

Material and methods
The group of patients
This study was performed on resected specimens
obtained from 126 patients who had undergone sur-
gical resection for primary sporadic colorectal cancer,
and from 114 patients who had undergone colonoscopy
at the Municipal Hospital in Jaworzno (Poland). All the
specimens were obtained with the consent of the pa-
tients. In all cases, a experienced pathologist reviewed
the haematoxylin and eosin (H + E) slides of the ade-
nomas or primary tumours to confirm the pathological
features.
The subject population of colorectal cancer patients
comprised 83 men and 43 women. The tumours of the
patients were classified histopathologically as adeno-
carcinoma according to the WHO grading system: grade
1 – 8 (6.349%) patients; grade 2 – 71 (56.349%) pa-
tients; grade 3 – 47 (37.301%) patients.
The population of patients with adenoma comprised
53 men and 61 women. The adenomatous polyps were
classified as tubular adenomas – 64 (56.140%) patients;
villous adenomas – 28 (24.561%) patients; and tubulo-
villous adenomas – 22 (19.298%) patients.
The design of the study was approved by the ethical
committee of the Jerzy Kukuczka Academy of Physical
Education in Katowice. The study was supported by
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Silesia.

Immunohistochemistry of MnSOD
protein
Paraffin-embedded, 4-μm-thick tissue sections
were stained for rabbit polyclonal anti SOD2 antibody
obtained from GeneTex (clone TF9-10-H10 from Amer-
ica Diagnostica). Deparaffinisation of all sections was
performed through a series of xylene baths, and re-
hydration was performed through graded alcohols. To
retrieve the antigenicity, tissue sections were treated
three times with microwaves in a 10 mM citrate buffer
(pH 6.0) for 5 min each. Subsequently, antigen retriev-
al sections were incubated with rabbit polyclonal an-
ti-SOD2 antibody (final dilution 1 : 400). The En-Vision
method (DAKO En-Vision Kit/Alkaline Phosphatase
detection system) was used according to the manu-
facturer’s instructions. The bound primary antibody
was detected using the New Fuchsin Substrate System
(DAKO A/S).

Immunohistochemical analysis
We graded the immunoreactivity by using a semi-
quantiative approach. Immunohistochemical reaction
for MnSOD was classified into four groups according to
the intensity of immunohistochemical reaction: 0, neg-
ative; 1, weak; 2, moderate; and 3, strong. The intensity
of immunohistochemical reaction on the surface epithelial
cells has been described as strongly positive. Diffuse
staining with the staining intensity weaker than that of
surface epithelial cells was characterised as moderately
positive. Faint or focal staining was described as weakly
positive. Heterogeneity was defined as the proportion
of cancer cells showing a positive reaction to the to-
tal number of cancer cells and was graded from 0 to
3 by assessment: 0 demonstrated negative staining,
1 represented less than 10%, 2 represented 10–50%, and
3 represented more than 50% of cancer cells with posi-
tive reaction. The results of intensity of staining and
heterogeneity were combined and scored as follows:
0 represented negative, 1 and 2 represented low, 3 and
4 represented moderate, and 5 and 6 represented high expression.

**Statistical analysis**

The relationship between MnSOD expression and clinicopathological variables were examined by the R: A Language and Environment for Statistical Computing (R Core Team, Vienna, Austria) using Pearson’s χ² test. The accepted level of statistical significance was \( p < 0.05 \).

**Results**

In normal colorectal mucosa MnSOD was predominantly localised in the basal cytoplasm of surface epithelial cells, in which MnSOD immunoreactivity was characterised as strong and granular (Figure 1 A). In some glandular epithelial cells of the crypts MnSOD expression was also observed. Its intensity was described as weak. Weak expression was also detected in muscularis mucosa and blood vessels in submucosa (Figure 1 B). The scattered inflammatory cells localised within lamina propria showed moderate and strong cytoplasmic expression of MnSOD.

In adenoma samples strong granular immunoreactivity was detected in neoplastic cells (Figures 1 C, D). In inflammatory cells within lamina propria expression was characterised as moderate or strong. The scattered fibroblast-like cells that were found in close proximity to neoplastic glands were also immunopositive. In those stromal cells, MnSOD immunoreactivity was characterised as strong and granular. Adenomas with high grade of dysplasia revealed a significantly higher expression of MnSOD in comparison to those with low grade of dysplasia (\( \chi^2 = 13.5429; df = NA; p = 0.0004998 \)). Moreover, adenomas with high grade of inflammatory infiltration revealed significantly higher expression of this enzyme (\( \chi^2 = 93.4386; df = 7; p = 2.2e-16 \)). There was no correlation between intensity of MnSOD immunoreactions and age, gender, localisation, and histological type of adenoma (Table I).

In adenocarcinoma samples, MnSOD expression was found in the cytoplasm of both stromal and cancer cells (Figures 1 E, F). Also, the walls of blood vessels showed positive immunoreactions, but the intensity was weak. The scattered cells with positive reaction were found in submucosa and fatty tissue as well. Immunohistochemical expression of MnSOD in adenocarcinoma samples was significantly correlated with histological grade of tumour (\( \chi^2 = 19.1451; df = NA; p = 0.0008496 \)), lymphovascular invasion (\( \chi^2 = 16.6624; df = 3; p = 0.0008292 \)), and regional lymph node involvement (\( \chi^2 = 12.9936; df = NA; p = 0.04248 \) (Table II)). There was no correlation between MnSOD immunoreactivity and age, gender, size of primary tumour, location, and depth of invasion.

**Discussion**

Numerous reports have implicated ROS and the activation of redox-sensitive signalling pathways as pivotal players in neoplasm development [12, 13]. Moreover, it has been reported that intrinsic antioxidant enzymes are vital to the regulation of oxidative stress within cells, and alterations of these enzymes are connected with cancer pathogenesis as well [14]. Of these, one of the primary cellular antioxidants SOD catalyses the conversion of superoxide to \( \text{H}_2\text{O}_2 \), which can then be removed by catalase, glutathione peroxidases, or peroxiredoxins. *In vitro* studies have shown that a number of cancer cell lines contain elevated levels of MnSOD and decreased levels of catalase, and that this disruption in steady-state level of \( \text{H}_2\text{O}_2 \) showed a correlation with increased metastasis, proliferation, and resistance to apoptosis. MnSOD-dependent production of \( \text{H}_2\text{O}_2 \) increased the expression of matrix-degrading metalloproteinases (MMP), which can alter the cancer microenvironment, forming a permissive conditions for metastatic disease [15, 16]. For example, Hempel *et al.* demonstrated that acquisition of metastatic phenotype was associated with a significant increase in the expression and activity of MnSOD and decreased activity of catalase. This, in turn, was associated with enhanced steady-state production of \( \text{H}_2\text{O}_2 \) in the 253J B-V cells, and with increased activity of MMP-9 and VEGF [17]. Liu *et al.* determined that MnSOD expression was consistently elevated in patients with tongue cancer. In such patients, expression of MnSOD was significantly higher in cases with lymph node metastases [18]. Several studies demonstrated increased expression of MnSOD in oesophageal [19–24], oral squamous cell carcinoma [25], and pancreatic cancer [26]. In some cases enhanced MnSOD expression was related to poor survival rate or recurrence. Nozoe *et al.* revealed that the proportion of lymph node metastasis in MnSOD-positive colorectal carcinomas was significantly higher than in MnSOD-negative carcinomas. Moreover, the survival rate in patients with MnSOD-positive carcinomas was worse than that in patients with MnSOD-negative carcinomas [27]. Similar findings have been detected in gastric [19, 28–32] and pancreatic cancer [25], and oral squamous cell carcinoma. The question arises regarding the reasons for enhanced activity of MnSOD in the metastatic stage of the disease. Dhar *et al.* suggested that reduced Sp1 binding to the MnSOD promoter is responsible for decreased MnSOD expression in the early stages of cancer development, whereas reduced p53 binding activity is responsible for restoration of MnSOD.
at later stages of cancer development. In this case, the balance between Sp1 and p53 plays an important role in regulating the MnSOD level during the phenotypic changes leading to aggressive growth. It has been suggested that increased MnSOD activity may protect cells against mitochondrial injury, thereby conferring...
Immunohistochemical assessment of mitochondrial superoxide dismutase (MnSOD) in colorectal premalignant and malignant lesions

In vitro studies also showed that elevated levels of MnSOD were correlated with acquisition of invasive abilities. It is generally accepted that the major process responsible for cell metastatic behaviour is epithelial-mesenchymal transition (EMT) [34]. Interestingly, transcription factors involved in EMT activation seem to be under the influence of MnSOD. Both Snail1 and Snail2 were highly expressed in metastatic UM1 cells in comparison to UM2 cells. Upon MnSOD knockdown in UM1 cells, the intracellular levels of H2O2 levels were decreased and the expression of Snail factors was inhibited. Moreover, the level of Snail factors was correlated with MMP-1 and ERK1/2. These results may indicate that MnSOD activates Snail signalling, increasing the same metastatic properties of cancer cells [18].

The results of our study remain in agreement with the findings mentioned above. We also revealed that samples of colorectal adenocarcinoma showed significantly higher immunoreexpression of MnSOD in comparison to adenomas and adjacent mucosa without any pathological lesions. High intensity of immunoreactions was detected in G3 tumours, which are known to be highly metastatic. Moreover, expression of MnSOD was correlated with lymphovascular invasion and regional lymph node involvement. Approximately 50% of patients with lymphovascular invasion showed strong expression of MnSOD, whereas only 15% from the negative group showed a strong pattern of MnSOD expression. As we discovered, regional lymph node involvement was also correlated with MnSOD positivity. The majority of patients with N2 status showed strong expression, whereas only 1 patient from the N0 group showed such strong reaction intensity.

MnSOD expression was also upregulated in the case of colorectal adenomas, which are known as precursor lesions of carcinoma. Adenomas with high grade of dysplasia have significantly higher level of MnSOD immunoreactivity. Only 2 patients from the group characterised with a high degree of dysplasia were negative, whereas 45% demonstrated strong expression. Similar results have been obtained in patients with strong infiltration of immune cells in lamina propria. Half of the cases revealed strong expression, whereas only 3 were described as negative. On the other hand, patients from the group characterised by weak infiltration did not show negative immunoreaction. Most of them showed moderate reaction and only 15 % revealed strong intensity. It is not surprising that inflammatory infiltration is an important factor that can influence MnSOD expression pattern. As revealed by studies, MnSOD is detected not only within cytoplasm of neoplastic cells but also in stromal cells, including macrophages and neutrophils. Accumulating phagocytic leukocytes gener-

Table I. Relation between MnSOD expression and clinicopathological variables in colorectal adenoma patients

| Variables                      | Total | Expression status, n (%) | P-value |
|--------------------------------|-------|--------------------------|---------|
|                                |       | Negative | Low | Moderate | High |       |
| Age < 50                       | 56    | 9 (16.07) | 25 (44.64) | 16 (28.57) | 6 (10.71) | 0.1009 |
| Age ≥ 50                       | 58    | 12 (20.69) | 16 (27.59) | 27 (46.55) | 3 (5.17) |         |
| Gender Male                    | 53    | 11 (20.75) | 16 (30.19) | 22 (41.51) | 4 (7.55) | 0.6647  |
| Gender Female                  | 61    | 10 (16.39) | 25 (40.98) | 23 (37.70) | 3 (4.92) |         |
| Location Proximal colon        | 60    | 10 (16.67) | 18 (30.00) | 29 (48.33) | 3 (5.00) | 0.07096 |
| Location Distal colon          | 30    | 5 (16.67)  | 12 (40.00) | 11 (36.67) | 2 (6.67) |         |
| Location Rectum                | 24    | 9 (37.50)  | 11 (45.83) | 3 (12.50)  | 1 (4.17) |         |
| Inflammatory infiltrate Weak   | 39    | 0 (0.00)   | 8 (20.51)  | 25 (64.10) | 6 (15.38) | < 0.001 |
| Inflammatory infiltrate Strong | 75    | 3 (4.00)   | 10 (13.33) | 21 (28.00) | 41 (54.67) |         |
| Degree of dysplasia Low grade  | 43    | 1 (2.33)   | 9 (20.93)  | 26 (60.47) | 7 (16.28) | 0.0004998 |
| Degree of dysplasia High grade | 71    | 2 (2.82)   | 17 (23.94) | 20 (28.17) | 32 (45.07) |         |
| Histological type of adenoma   |       |            |            |            |            |
| Tubular                        | 64    | 11 (17.19) | 19 (29.69) | 31 (48.44) | 3 (4.69) | 0.1719 |
| Villous                        | 28    | 5 (17.86)  | 10 (35.71) | 11 (39.29) | 2 (7.14) |         |
| Tubulo-villous                 | 22    | 7 (31.82)  | 11 (50.00) | 3 (13.64)  | 1 (4.55) |         |

Gastroenterology Review 2016; 11 (4)
### Table II. Relation between MnSOD expression and clinicopathological variables in colorectal cancer patients

| Variables                  | Total number of cases | Expression status, n (%) | P-value |
|----------------------------|-----------------------|--------------------------|---------|
|                            |                       | Negative | Low  | Moderate | High   |         |
| **Age:**                   |                       |           |      |          |        |         |
| < 50                       | 33                    | 10 (30.30)| 4    | (12.12)  | 10 (30.30)| 9 (27.27)| 0.1654 |
| ≥ 50                       | 93                    | 20 (21.51)| 5    | (5.38)   | 23 (24.73)| 45 (48.39)|         |
| **Gender:**                |                       |           |      |          |        |         |
| Male                       | 83                    | 17 (20.48)| 6    | (7.23)   | 24 (28.92)| 36 (43.37)| 0.2529 |
| Female                     | 43                    | 16 (37.21)| 3    | (6.98)   | 9 (20.93)| 15 (34.88)|         |
| **Size of primary tumour:**|                       |           |      |          |        |         |
| < 5 cm                     | 44                    | 11 (25.00)| 0    | (0.00)   | 13 (29.55)| 20 (45.45)| 0.1844 |
| ≥ 5 cm                     | 82                    | 22 (26.83)| 8    | (9.76)   | 19 (23.17)| 33 (40.24)|         |
| **Location of tumour:**    |                       |           |      |          |        |         |
| Proximal colon             | 31                    | 3 (9.68) | 3    | (9.68)   | 10 (32.26)| 15 (48.39)| 0.2784 |
| Distal colon               | 83                    | 25 (30.12)| 7    | (8.43)   | 17 (20.48)| 34 (40.96)|         |
| Rectum                     | 12                    | 3 (25.00) | 2    | (16.67)  | 4 (33.33) | 3 (25.00) |         |
| **Histological grade:**    |                       |           |      |          |        |         |
| G3                         | 47                    | 2 (4.26) | 3    | (6.38)   | 10 (21.28)| 32 (68.09)| 0.008496|
| G2                         | 71                    | 7 (9.86) | 4    | (5.63)   | 17 (23.94)| 43 (60.56)|         |
| G1                         | 8                     | 2 (25.00) | 3    | (37.50)  | 3 (37.50) | 0 (0.00)  |         |
| **Depth of invasion:**     |                       |           |      |          |        |         |
| T1                         | 6                     | 2 (33.33)| 1    | (16.67)  | 2 (33.33)| 1 (16.67) | 0.1159 |
| T2                         | 30                    | 6 (20.00)| 7    | (23.33)  | 9 (30.00)| 8 (26.67) |         |
| T3                         | 68                    | 9 (13.24)| 15   | (22.06)  | 21 (30.88)| 23 (33.82)|         |
| T4                         | 22                    | 1 (4.55) | 9    | (40.91)  | 11 (50.00)| 1 (4.55)  |         |
| **Lymphovascular invasion:**|                       |           |      |          |        |         |
| Positive                   | 85                    | 10 (11.76)| 15   | (17.65)  | 19 (22.35)| 41 (48.24)| 0.0008292|
| Negative                   | 41                    | 11 (26.83)| 6    | (14.63)  | 18 (43.90)| 6 (14.63) |         |
| **Regional LN involvement:**|                       |           |      |          |        |         |
| N2                         | 83                    | 21 (25.30)| 7    | (8.43)   | 21 (25.30)| 34 (40.96)| 0.04248|
| N1                         | 31                    | 1 (3.23) | 3    | (9.68)   | 11 (35.48)| 16 (51.61)|         |
| N0                         | 12                    | 3 (25.00)| 2    | (16.67)  | 6 (50.00)| 1 (8.33)  |         |

Kruidenier et al. demonstrated that they also contain high MnSOD levels. Interestingly, we did not observe any correlation between MnSOD expression and histological type of adenoma [36]. It is generally accepted that villous polyps in particular are considered high risk. With respect to malignancy, it was found that only 4% of tubular adenomas but 40% of villous adenomas developed into carcinoma [37]. It may take about 10 years for an adenomatous polyp to transform into cancer [38].
cells to utilise oxidative phosphorylation for maximum energy production to increase cell survival and growth. In the early stages of tumorigenesis, lower MnSOD content may be a threat to mitochondrial function, leading to a shift to glycolysis in cancer cells. Alternatively, it has been proposed that glycolytic metabolism arises as an adaptive response to hypoxic conditions during the early stages of tumour development because it permits ATP generation even in the absence of oxygen. Thus, excess lactate production may further drive the evolution of glycolytic metabolism for energy production [39]. It has been also proposed that aerobic glycolysis in tumour cells may require rapid influx of substrates through glycolysis, allowing the effective shunting of glucose or fructose as a source of carbon energy production [40–42]. However, because of the consistently increasing demand for energy during the aggressive growth of cancer cells, particularly in the later stages of carcinogenesis, the energy supply from glycolytic processes may not be sufficient. In this situation, it is possible that aggressive cancer cells switch back from glycolysis to oxidative phosphorylation for their increased energy requirements. For this purpose, cancer cells need to increase the efficiency of mitochondrial function [9].

Conclusions

The results of our study have demonstrated that development of colorectal cancer is connected with increased expression of MnSOD both in adenoma and adenocarcinoma stages. Samples of adenocarcinoma with G2 and G3 grade showed significantly higher levels of immunohistochemical expression of this antioxidant enzyme. Moreover, patients with the presence of lymphovascular invasion and higher degree of regional lymph node status were also characterised by higher level of MnSOD expression. The samples of adenoma were characterised by higher level of MnSOD expression in comparison to normal mucosa as well. Interestingly, there was no significant correlation between expression and histological type of adenoma.

Conflict of interest

The authors declare no conflict of interest.

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