Objectives: A comprehensive understanding of the role of PON enzymatic activities may play an important role in the etiology and prevention of many cancers. PON1 is known as a potent antioxidant that scavenges free radicals in the human body. The enzymatic activities of paraoxonase (PON1) and mitogen-activated protein kinase (MAPK) signalling pathways in colorectal cancer are being investigated to determine whether they hold promise for novel diagnostic or therapeutic applications in colorectal cancer.

Methods: HT-29 colon cancer cell lines and CCD-18Co colon cell lines were used. B-Raf, p-B-Raf, ERK, and p-ERK proteins involved in MAPK signalling pathways and serum levels of PON1 were detected and analyzed by the Western blotting method.

Results: The levels and activity of PON1 enzyme were significantly decreased in HT-29 cells compared to CCD-18Co cells (p=0.0173 and p=0.0281, respectively). The levels of p-B-Raf and p-ERK, which activates the MAPK signalling pathway, were significantly increased in HT-29 cells (p=0.0037 and p=0.0074, respectively).

Conclusions: A positive correlation was found between PON1 level and PON1 enzyme activity (p=0.008). A negative correlation was also found between PON1 enzyme activity and p-B-Raf/p-ERK protein levels (p=0.060 and p=0.037, respectively).

It is suggested that the increase of proteins involved in the MAPK signaling pathway in cancer cells is caused by the decreasing serum levels of PON1 and enzymatic activity of PON1.

Keywords: antioxidant; colon cancer; ERK pathway; oxidative stress; paraoxonase-1
functions show increased or decreased activity by transforming into oncogenic signalling pathways in cancer cells. The MAPK pathway is overactive in approximately 30% of human tumors and 40% of CRCs [6, 7].

Oxidative stress occurs when the balance of oxidation in our body is disturbed for any reason. As we age, the production of reactive oxygen species (ROS) and free radicals increases. The harmful effects of ROS, produced during normal cellular metabolism, are eliminated by antioxidant systems. However, when ROS is not neutralized, oxidative stress occurs, which may play a role in the development and progression of cancer [8, 9].

Human antioxidants originate from ‘endogenous’ produced naturally by the body or ‘exogenous’ taken as an external supplement. Paraoxonase (PON) enzymes are a family of antioxidant enzymes that are among the endogenous free radical scavenging systems and have anti-inflammatory effects. The PON enzyme family consists of three known members, PON1, PON2, and PON3, and all three prevent oxidative stress. The calcium-dependent PON1 and PON3 are synthesized in the liver, bound to high-density lipoprotein (HDL), transported to cell membranes in various tissues and organs, and distributed [10, 11]. Alterations in PON enzyme activity and expression lead to inflammation with oxidative stress. Long-term intense oxidative stress and inflammation can both lead to carcinogenesis and provide an ideal environment for tumor cell growth [12]. It is suggested that PON1 activity and/or its polymorphisms may be a leading biomarker for carcinogenesis in pancreatic, liver, brain, breast, gastrointestinal, prostate, and ovarian cancers [13]. There are some studies in the literature on the role of both PON enzymes and MAPK pathways in the development of CRC. However, these studies aim to explain the role of both parameters independently in CRC carcinogenesis. No study was found that investigated the effects of these two parameters on each other in colorectal carcinogenesis. This study was carried out to test the role of the PON1 enzyme and MAPK pathway in the formation of colon cancer and to examine the interaction between PON1 and MAPK pathways in the cancerization process.

Materials and methods

Cell culture

The colon adenocarcinoma cell line HT-29 (ATCC HTB-38) and the healthy colon fibroblast cell line CCD-18Co (ATCC CRL-1459) were used in our study. In line with the manufacturer’s recommendation, HT-29 cells were grown in Dulbecco’s Modified Eagle Medium, and CCD-18Co cells used for control purposes were grown in Eagle’s Minimum Essential Medium. Cells were cultured in a medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 5% CO2 and 37°C in 25 cm2 (T25) flasks and a cell culture incubator under sterile conditions. When the cells were 90% confluent, 200 µL of lysis buffer was added (The lysis buffer was prepared by adding distilled water to the solution containing 50 mM Tris, 150 mM NaCl, 1% NP-40, and 1 mL of proteinase inhibitor factor to make the final volume pH-8). Cells homogenized on ice with a cell scraper were sonicated three times. The lysates obtained by collecting the supernatant of the cells centrifuged at 4°C and 6179 × g for 15 min were stored at –20°C until the time of assay.

Measurements of PON1 enzyme activity

The enzyme activity of PON1 from the prepared lysates was determined spectrophotometrically [14, 15]. For this purpose, basal activity buffer (100 mM Tris–HCl containing 2 mM CaCl2) and substrate (2 mM paraoxon solution) were first prepared. In the next step, the basal activity buffer, the freshly prepared substrate solution, and the prepared lysates were added to the spectrophotometer cuvette. The absorbance values of the test samples were determined at 37°C at 412 nm. Then, the PON1 enzyme activities (EU) were calculated using the following formula [16].

\[
EU = \frac{[\text{reaction volume (mL)} \times \text{dA/dT} \times 1,000]}{[\text{d cm}] \times \text{enzyme volume (mL)} \times d \text{ (cm)}}
\]

Determination of protein levels

The PON1 level and B-Raf, p-B-Raf, ERK, and p-ERK protein levels in the MAPK signalling pathway were analyzed by SDS-PAGE and Western blot.

SDS page

Protein concentrations in the samples were measured by Nanodrop before proceeding to the gel run. 2× Laemmli sample buffer was added to the prepared lysates and vortexed. Protein denaturation was achieved by boiling the mixture at 96°C in the heating block for 8–10 min 4–12% bis-tris gel was used for the electrophoresis run. The comb on the walls of the prepared gel was removed, and each well was washed with a syringe containing run buffer (40 mL NuPAGE run buffer and 760 mL distilled water). One of the wells was loaded with a 5 µL ladder, while the others were loaded with 20 µL samples each. After loading was completed, the tank was sealed, the electrodes were connected, and the power supply was set to 100 V, 120 A, and the run was performed for 100 min.

Western blot

The iBlot dry blotting protocol was used for protein transfer, which has higher efficiency than the wet and semi-dry transfer methods [17]. The gel transferred to the membrane was stained with Ponceau S dye to verify that the proteins were transferred to the membrane. A milk powder solution was used to prevent unnecessary binding by closing large antigenic regions on the membrane. After blocking, the membrane was washed with PBS-Tween20 for 10–15 min and incubated with the primary antibodies, whose names and dilution rates are listed in Table 2 overnight at +4°C in a shaker (130 rpm). The membrane incubated with the primary antibodies was washed three times with PBS-Tween20 solution for 10–15 min. It was then incubated with HRP-conjugated
secondary antibodies for 1 h at room temperature in a shaker (Table 2). After completion of the process, the membrane was washed with PBS-Tween20 for 15–20 min.

The membrane was treated with an enhanced luminol-based chemiluminescent agent (Pierce™ ECL substrate), and imaging was performed using the UVP ChemiDoc-it2 imager instrument. The band images corresponding to the protein levels of PON1, B-Raf, p-B-Raf, ERK, and p-ERK in the cell lines were calculated by normalization with α-tubulin in the Image J program (National Institutes of Health, USA). The experimental steps were performed with three replicates, and the data obtained from these replicates were used for the statistical analyses.

Statistical analysis and evaluation of results

Statistical analyzes and calculations were performed using the GraphPad Prism 9 program for the Windows package program (GraphPad Software Inc., San Diego, CA). Changes in protein concentrations and enzyme activity results in cell lines were analyzed with t-tests for two independent samples. The relationships between the PON1 level and enzyme activity and the levels of proteins involved in the MAPK pathway were examined using Pearson correlation analysis [18]. In our study, the statistical significance level was accepted as p<0.05.

Results

Measurement of PON1 level and PON1 enzyme activity in cell lines

The PON1 expression level calculated by Western Blot analysis significantly decreased in the HT-29 cell line (p=0.0173) (Figure 1A). In addition, PON1 enzyme activity determined by spectrophotometry was significantly decreased in HT-29 cells compared to CCD-18Co cells (p=0.0281) (Figure 1B). According to our findings, a significant, very high positive correlation was found between PON1 level and PON1 enzyme activity (r=0.927, p=0.008). In cases where PON1 expression decreased, PON1 enzyme activity was also decreased (Table 1).

Determination of active and inactive expression levels of B-Raf and ERK proteins involved in the MAPK signalling pathway

B-Raf protein levels were decreased in HT-29 cancer cells compared to healthy CCD-18Co cells. However, this decrease was not significant (p=0.7707) (Figure 2A). Also, the level of ERK was significantly decreased in HT-29 cancer cells compared to healthy cells (p=0.0004) (Figure 2C). In addition, the levels of phosphorylated B-Raf (p-B-Raf) and phosphorylated ERK (p-ERK) were significantly increased in HT-29 cancer cells compared to healthy cells (p=0.0037 and p=0.0074, respectively) (Figure 2B and D).

Measurement of correlation between PON1 enzyme and MAPK pathway proteins

In our study, a significantly high negative correlation was found between PON1 levels and p-B-Raf and p-ERK levels (r=−0.893, p=0.016; r=−0.840, p=0.036), respectively. According to these results, the protein levels of p-B-Raf and p-ERK, which are responsible for the activation of the signalling pathway, significantly increased in the cases where the PON1 level decreased. In addition, an insignificant high negative correlation was found between PON1 enzyme activity and p-B-Raf level (r=−0.793, p=0.060). Additionally, a statistically
Table 1: Correlation matrix showing the relationship between PON1 level and PON1 enzyme activity in colon cells.

<table>
<thead>
<tr>
<th></th>
<th>PON1 levels</th>
<th>PON1 enzyme activity</th>
<th>B-RAF protein levels</th>
<th>p-B-RAF protein levels</th>
<th>ERK protein levels</th>
<th>p-ERK protein levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1 levels</td>
<td>r=1.00</td>
<td>r=0.927&lt;sup&gt;a&lt;/sup&gt;</td>
<td>r=0.491</td>
<td>r=−0.893&lt;sup&gt;b&lt;/sup&gt;</td>
<td>r=0.926&lt;sup&gt;b&lt;/sup&gt;</td>
<td>r=−0.840&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PON1 enzyme activity</td>
<td>r=0.927&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p=0.008</td>
<td>p=0.323</td>
<td>p=0.016</td>
<td>p=0.008</td>
<td>p=0.036</td>
</tr>
<tr>
<td>B-RAF protein levels</td>
<td>r=0.491</td>
<td>p=0.008</td>
<td>p=0.581</td>
<td>r=−0.282</td>
<td>p=0.060</td>
<td>p=0.008</td>
</tr>
<tr>
<td>p-B-RAF protein levels</td>
<td>r=0.323</td>
<td>p=0.581</td>
<td>p=0.588</td>
<td>r=−0.282</td>
<td>p=0.725</td>
<td>p=0.548</td>
</tr>
<tr>
<td>ERK protein levels</td>
<td>r=0.926&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p=0.016</td>
<td>p=0.060</td>
<td>r=−0.793</td>
<td>p=0.102</td>
<td>p=0.036</td>
</tr>
<tr>
<td>p-ERK protein levels</td>
<td>r=−0.840&lt;sup&gt;a&lt;/sup&gt;</td>
<td>p=0.008</td>
<td>p=0.008</td>
<td>r=−0.838&lt;sup&gt;a&lt;/sup&gt;</td>
<td>p=0.012</td>
<td>p=0.008</td>
</tr>
<tr>
<td></td>
<td>r=0.036</td>
<td>p=0.037</td>
<td>p=0.548</td>
<td>r=−0.311</td>
<td>p=0.840&lt;sup&gt;a&lt;/sup&gt;</td>
<td>r=1.000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant at p<0.05 level, <sup>b</sup>Significant at p<0.01 level, significant at p<0.001 level. Pearson correlation coefficient (r): 0.90–1.00 (−0.90 to −1.00), very high positive (negative) correlation; 0.70–0.90 (−0.70 to −0.90), high positive (negative) correlation; 0.50–0.70 (−0.50 to −0.70), moderate positive (negative) correlation; 0.30–0.50 (−0.30 to −0.50), low positive (negative) correlation; 0.00–0.30 (0.00 to −0.30), negligible positive (negative) correlation.

Table 2: Primary and secondary antibodies are used in the blotting and dilution rates of these antibodies.

<table>
<thead>
<tr>
<th></th>
<th>Company (Catalog#)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PON1 polyclonal antibody</td>
<td>Thermo Fisher (PA-115780)</td>
<td>1/3,000</td>
</tr>
<tr>
<td>Anti B-RAF antibody</td>
<td>St John’s Lab. (STJ95353)</td>
<td>1/500</td>
</tr>
<tr>
<td>Anti p-BRAF antibody</td>
<td>St John’s Lab. (STJ22042)</td>
<td>1/500</td>
</tr>
<tr>
<td>ERK1/2 monoclonal antibody</td>
<td>Thermo Fisher (ERK-7D8)</td>
<td>1/500</td>
</tr>
<tr>
<td>Anti p-ERK1/2 antibody</td>
<td>St John’s Lab. (STJ91357)</td>
<td>1/500</td>
</tr>
<tr>
<td>Tubulin alpha antibody</td>
<td>Affibiotec (AP7010)</td>
<td>1/5,000</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat-anti-mouse IgG (H+L), HRP conjugate</td>
<td>Advansta (R-05071-500)</td>
<td>1/20,000</td>
</tr>
<tr>
<td>Goat-anti-rabbit IgG (H+L), HRP conjugate</td>
<td>Advansta (R-05072-500)</td>
<td>1/20,000</td>
</tr>
</tbody>
</table>

Figure 2: Variation of protein levels normalized with α-tubulin in cell lines using western blot band images. (A) B-Raf levels, (B) p-B-Raf levels, (C) ERK levels, (D) p-ERK levels.

ns: non-significant , * significant at p<0.05 level, ** significant at p<0.01 level, *** significant at p<0.001 level

Discussion

Studies conducted in recent years have provided new insights into the genesis and development of CRC at the biochemical and molecular levels. Oxidative stress and the accumulation of ROS have been associated with an increased risk of several types of cancer. Today, low activity of the high negative correlation was found between PON1 enzyme activity and p-ERK level (r=−0.838, p=0.037). According to these results, in the cases where PON1 enzyme activity decreases, an insignificant increase in p-B-Raf levels, which plays a role in MAPK pathway activation, was detected, and an excellent increase in p-ERK levels was observed (Table 1).
enzyme PON1 in serum, which causes oxidative stress, is thought to play a role in carcinogenesis. According to studies in the literature, both PON1 enzyme activity and PON1 levels (arylesterase and/or paraoxonase activity) decrease in CRC, and therefore, PON1 is recommended as a biomarker for CRC patients [19–21].

Defects in the capacity of the antioxidant system and altered PON1 enzyme activity and/or PON1 levels are thought to play a role in the pathogenesis of CRC. According to recent studies in the literature, the levels of PON1 enzyme and paraoxonase activity are decreased in CRC patients with one or two exceptions [19, 20, 22]. In our study, it was found that PON1 level and PON1 enzyme activity were significantly decreased in HT-29 colon cancer cells compared with healthy CCD-18Co colon cells. These results support the studies in the literature. According to our findings, there is a very high positive correlation between PON1 level and PON1 enzyme activity in cancer cells. Decreased PON1 level in the HT-29 cell line resulted in decreased PON1 enzyme activity.

Overactivity of the MAPK pathway is involved in many key processes of colon cancer development. Inhibition of this pathway inhibits tumor angiogenesis and halts tumor growth and metastasis. According to studies in the literature, abnormal activation of the MAPK pathway leads to differentiation of the intestinal epithelium and plays a role in the oncogenic behavior of CRC. Approximately 44 % of CRCs exhibit dysregulation of the MAPK pathway due to a K-Ras mutation and 10 % due to a B-Raf mutation [23, 24]. In our study, the active and inactive levels of B-Raf involved in the MAPK signaling pathway were analyzed in HT-29 colon cancer cells and CCD-18Co healthy colon cells. It was found that the p-B-Raf protein level was significantly increased in HT-29 cancer cells. Another protein of particular importance in the generation of the cellular response in the MAPK signaling system is ERK. ERK is the final step of the signalling cascade, and activated ERK (p-ERK) migrates into the nucleus and mediates the cellular response through hundreds of transcription factors. In our study, it was found that ERK protein levels significantly decreased and p-ERK levels significantly increased in HT-29 cancer cells. These increases in p-B-Raf and p-ERK levels suggest that the MAPK signalling pathway is over-activated in the HT-29 cancer cell line.

In our study, we investigated the effects of the endogenous antioxidant enzyme PON1 on the MAPK signaling pathway in both HT-29 cancer cell lines and healthy CCD-18Co cell lines. Our results showed a significant and high negative correlation between the level of PON1 and the levels of the proteins p-B-Raf and p-ERK. We also observed an insignificant but high negative correlation between PON1 enzyme activity and p-B-Raf protein. In addition, a high negative correlation was observed between PON1 enzyme activity and p-ERK protein levels.

In our study, the level and enzyme activity of PON1, an endogenous antioxidant, decreased in HT-29 cancer cells compared with healthy cells. This suggests that the disturbed oxidative balance resulting from the loss of antioxidants may be responsible for the abnormal activation of the MAPK pathway in cancer cells.

Further studies are needed to explain the physiological role of PON1 in the development of CRC and also to uncover its relationship with MAPK signaling pathways. Studies in patient groups that consider the localization and stage of colorectal cancer may provide a broader perspective.

Acknowledgments: The authors would like to thank Dr. Tuğba Keşer Uysal for her support during the laboratory process.

Research ethics: Not applicable.

Informed consent: Not applicable.

Author contributions: All authors have taken responsibility for the entire content of this manuscript and approved its submission.

Competing interests: The authors have no conflicts of interest to disclose.

Research funding: This research was supported by Ankara Yıldırım Beyazıt University Scientific Research Projects Unit under project number 2196.

Data availability: The raw data can be obtained on request from the corresponding author.

References


