Quantitative analysis of \textit{HMGA1} gene expression in human colorectal cancer

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\textbf{Abstract}
\textit{HMGA1} gene encoding high-mobility group A1 proteins (HMGA1) seems to play an important role in colorectal carcinogenesis. Our previous work revealed strong statistical correlations between \textit{HMGA1} gene expression (estimated by reverse transcriptase-PCR method) and advanced stages of colon cancers. On the basis of these results cases with the presence of \textit{HMGA1} gene were taken for quantitative analysis by means of real-time PCR method. In this group \textit{HMGA1} gene expression level was compared with some histological features, grading and clinical staging of investigated neoplasms. The level of \textit{HMGA1} mRNA expression was statistically higher in more advanced tumors with lymph nodal (N1-N2) and distant metastases (M1) and without lymphocytic infiltration in tumor tissue. Associations between other parameters: age, gender, tumor localization, histological type and the level of \textit{HMGA1} gene expression were not found. The results of this work proved that not only the presence of \textit{HMGA1} gene expression but also its high levels can be observed in advanced cases of colon cancers. They confirm a role of \textit{HMGA1} gene in the process of metastasis formation that could potentially be useful in clinical practice.

\textbf{Keywords}: colorectal cancer, \textit{HMGA1} gene, real-time PCR, metastasis.

\section*{INTRODUCTION}

The \textit{HMGA1} gene, located at chromosomal locus 6p21 in humans, encodes nonhistone proteins, resulting from alternative splicing, which participate in different nuclear processes. They are characterized by three DNA binding domains called AT hooks which allow them to bind to the minor grooves of AT-rich sequences regions in the DNA helix. They have little secondary structure in solution but assume distinct conformations when bound to substrates such as DNA or other proteins. These proteins are engaged in many cellular processes, including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the metastatic progression of cancer cells (Balcerczak \textit{et al.}, 2005). \textit{HMGA1} gene expression is high in non-differentiated embryonal tissues (Chiappetta \textit{et al.}, 1996) and very low or even undetectable in differentiated somatic cells (Lundberg \textit{et al.}, 1989; Giancontti \textit{et al.}, 1985; 1987), which may suggest its crucial role in cell growth and differentiation. High \textit{HMGA1} gene expression or its protein product is characteristic for transformed cells (Johnson \textit{et al.}, 1989; 1990) and was observed in many types of cancers e.g. thyroid (Chiappetta \textit{et al.}, 1995; 1998; Dal-Cin \textit{et al.}, 1999), neuroblastic (Giannini \textit{et al.}, 2000), breast (Flohr \textit{et al.}, 2003), prostate (Bussemakers \textit{et al.}, 1991; Tamimi \textit{et al.}, 1993; 1996), pancreas (Abe \textit{et al.}, 2002), ovarian Masciullo \textit{et al.}, 2003), cervix (Bandiera \textit{et al.}, 1998), lung (Kettunen \textit{et al.}, 2004), colon (Fedele \textit{et al.}, 1996; Kim \textit{et al.}, 1999; Chiappetta \textit{et al.}, 2001; Abe \textit{et al.}, 1999; Fedele \textit{et al.}, 1996; Balcerczak \textit{et al.}, 2003), gastric (Lee \textit{et al.}, 2002) and all leukemias (Pierantoni \textit{et al.}, 2003). Strong immunohistochemical reaction with anti-HMGA1 antibodies is usually observed in primary carcinoma tissue, and weak reaction for
tumors with low malignant potential, whereas, lack of reaction was noticed for healthy control tissue (Masciullo et al., 2003; Kettunen et al., 2004; Fedele et al., 1996; Kim et al., 1999; Chiappetta et al., 2001; Lee et al., 2002).

Recently, it was shown that HMGA1 overexpression could be a good marker of liver metastases Chuma et al., 2004). Its higher level was characteristic for MDA-MB-231 breast cancer cells that possess strong metastatic ability Liu et al., 1999). Positive immunohistochemical reaction with anti-HMGA1 antibodies was also observed for intraductal papillary mucinous tumors of the pancreas but only in cases showing growing features with invasive character Abe et al., 2002). These observations were further confirmed by others. Chen et al 2004 have shown that HMGA1 expression was associated with malignant progression in Barrett’s metaplasia. Liau et al., 2006 have proven that HMGA1 expression is a determinant of cellular invasiveness and metastasis in pancreatic cancer.

Our recent study indicated HMGA1 gene expression in 63% (51 out of 81) of colon cancer cases studied and its expression was connected with poor prognosis for the patient (Balcerczak et al., 2003). There were statistically significant dependences between HMGA1 gene expression and the presence of tumor cells in lymph nodes and distant metastases. These data confirmed the significant role of HMGA1 in colon cancer development and metastasis formation.

In the present paper real-time PCR technique was used to quantitatively analyze a total of 63 HMGA1 positive cases, 51 from our previous study (Balcerczak et al., 2003) and 12 additional colon cancer cases. The level of expression was compared with some histological features, grading and clinical staging to clarify the potential role of HMGA1 as a marker of metastasis.

**MATERIALS AND METHODS**

**Tissues**

Colon cancer cases (n=109) were obtained from patients operated on in the Oncological Centre of Lodz, Poland under the license of the local ethical committee (KE/286/05). These cases were analyzed by multiplex reverse transcriptase PCR (Balcerczak et al., 2003). The HMGA1 positive human colon cancer cases (n=63) were further analyzed quantitatively. This group consist of 30 women (mean age 60.5 ±5) and 33 men (mean age 62.5 ±5). The analyzed carcinomas were histologically classified as tubular adenocarcinoma (n=55) and mucinous adenocarcinoma (n=8).

**RNA isolation**

RNA was isolated by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on RNA isolation methodology developed earlier (Chomczyński & Sacchi, 1987).

**Real time quantitative analysis of HMGA1**

For quantitative analysis all RNA samples were treated with DNase (Sigma) to remove genomic DNA. RNA was transcribed into cDNA using Enhanced Avian HS RT-PCR Kit (Sigma). Real-time PCR was performed using iCycler (Bio-Rad) and SYBR Green Jump™ Start Tag ReadyMix™ (Sigma) according to manufacturer’s instructions.

The HMGA1 primer set 5’-GGCACTGAGAAGCGGGCGG-3’ (forward) and 5’CCCTTGTTTTTTGCTTCCCCTT-3’ (reverse) and conditions used in the assay were developed by others (Chiappetta et al., 1996). The following reagents were added to the proper plate for thermocycling: 25 μl Jump Start Tag Ready Mix, 0.5 μl reference dye, 1 μl of forward primer (final concentration 0.2 uM), 1 μl of reverse primer, 2 μl Magnesium Chloride (final concentration 25 mM), 5 μl template cDNA, and water (final volume 50 μl.) Cycling parameters were hot starting at 98°C for 5 min, followed by 2 min initial denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C, 1 min annealing at 54°C and 3 min extension at 72°C, followed by 7 min final extension at 72°C. To standardize the amount of RNA, the expression of β-actine gene was quantified in each sample using 5’-GAGGGCGCGGCCCCAGGCAACA-3’ (forward); 5’-CTCCTTAATGTACGAGGATTTC-3’ (reverse) primer set. The DNA level was monitored by measuring the increased fluorescence of SYBR Green through the PCR cycles and for analysis estimated at the threshold cycle. The final results are given as a non-relative ratio, because experiments with HMGA1
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and β-actine were done not as a multiplex, but in the separated tubes during the same PCR. The level of HMGA1 expression was calculated on the basis of standard curve (units used for preparing curve ng/ul). A standard curve for HMGA1 determination was obtained by plotting known quantities of genomic HMGA1 DNA selected in our previous study. Then data were transformed into Microsoft Excel. Measurements for all samples were carried out in triplicate.

Statistics

Statistical analysis was prepared on the basis of U Mann - Whitney test.

RESULTS

The level of HMGA1 gene expression was determined quantitatively by real-time PCR technique in colon cancer cases (n=63) which showed the expression of investigated gene in quality analysis. Clinicopathological profiles of investigated cases are summarized in Table 1.

The obtained quantitative results were compared with several clinicopathological parameters such as depth of tumor invasion (T), lymph node metastases (N), distant metastases (M) (TNM classification).

The level of HMGA1 gene expression in more advanced tumors (T3 and T4, deep wall penetration) was 7.14 ± 4.80 ng/μl, while in the T1-T2 group a lower level of expression was recorded (6.87 ± 5.25 ng/μl). However, difference between the levels in the both groups was not statistically significant.

The level of HMGA1 gene expression was analyzed in cases without and with presence of cancer cells in lymph node. In cases without invasion to lymph nodes the level was determined as 5.31± 4.13 ng/μl, whereas in the cases with tumor cells in lymph nodes (N1-N2) as 8.38± 5.06 ng/μl. These data revealed significant statistical difference (p=0.007, U Mann - Whitney test), Fig. 1.

In carcinomas with distant metastases the levels of HMGA1 expression were two times higher (10.80 ± 4.46 ng/μl) than in the group of cancers without distant metastases (4.92± 3.73 ng/μl). This difference was statistically significant (p=0.000002, U Mann - Whitney test), Fig. 2.

We also compared the levels of HMGA1 between the cases with presence and absence of lymphocytes in tumor tissue. In the group of cases without lymphocytes in tumor tissue the mean level was estimated as a 8.40 ± 5.30 ng/μl,
but in the group of cancers with the presence of lymphocytes the mean level was lower and reached value 5.00 ± 3.32 ng/μl. This difference was statistically significant (p=0.016, U Mann - Whitney test), Fig.3, Table 2.

Table 1: Clinicopathological profiles of investigated colon cancer cases.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Number of cases</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of tumor invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1, T2</td>
<td>15</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T3, T4</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>16</td>
<td>0.007</td>
</tr>
<tr>
<td>N1-2</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>44</td>
<td>0.000002</td>
</tr>
<tr>
<td>M1</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Vessel invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Involved</td>
<td>41</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Not involved</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>25</td>
<td>0.016</td>
</tr>
<tr>
<td>Absence</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Statistical dependences between hmgal mRNA levels and clinicopathologic features in colon cancer cases

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Number of cases</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade of malignancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cases</td>
<td>Mean</td>
<td>Standard error</td>
</tr>
<tr>
<td>G1</td>
<td>10</td>
<td>6,014 (1.74 – 14.68)*</td>
</tr>
<tr>
<td>G2</td>
<td>36</td>
<td>6,723 (1.52 – 19.6)*</td>
</tr>
<tr>
<td>G3</td>
<td>17</td>
<td>8,372 (2.46 – 19.06)*</td>
</tr>
</tbody>
</table>

Table 3: The HMGAl expression level in relation to tumor localization

<table>
<thead>
<tr>
<th>Tumor localization</th>
<th>Number of cases</th>
<th>Mean (ng/μl)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal carcinomas</td>
<td>18</td>
<td>7,456 (1.74 – 17.08)*</td>
<td>1,348</td>
</tr>
<tr>
<td>Colon carcinomas</td>
<td>45</td>
<td>6,960 (1.52 – 19.6)*</td>
<td>0,747</td>
</tr>
</tbody>
</table>

* - Bracketed figures give min – max range

We did not find any statistically significant difference between the mean level of HMGAl gene expression in cases with and without vessel invasion (Table 2). There were also no statistically significant differences between the gender, age, histological type, localization of the tumor (Table 3) and the level of HMGAl gene expression.

According to histological grade all analyzed colon carcinoma cases were divided into 3 groups (low, moderate, and high malignancy neoplasms). The levels of HMGAl gene expression were growing with the grade of malignancy but these differences were not statistically significant (Table 4).

Table 4: The HMGAl gene expression level depending on grade of malignancy of colon cancer cases

<table>
<thead>
<tr>
<th>Grade of malignancy</th>
<th>Number of cases</th>
<th>Mean (ng/μl)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>10</td>
<td>6,014 (1.74 – 14.68)*</td>
<td>1,367</td>
</tr>
<tr>
<td>G2</td>
<td>36</td>
<td>6,723 (1.52 – 19.6)*</td>
<td>0,899</td>
</tr>
<tr>
<td>G3</td>
<td>17</td>
<td>8,372 (2.46 – 19.06)*</td>
<td>1,258</td>
</tr>
</tbody>
</table>

* - Bracketed figures give min – max ran

Comparison of HMGAl gene expression level with pTNM staging showed that more advanced cases (stages III and IV) revealed statistically significant higher levels (7.86 8.44 ± 4.91 ng/μl) than stages I and II (5.06 4.93 ± 4.11 ng/μl) - p=0.001, U Mann – Whitney test Table 5.

Table 5: The HMGAl gene expression level in relation to the clinical stage of colon cancer cases

<table>
<thead>
<tr>
<th>Clinical stage based on pTNM</th>
<th>Number of cases</th>
<th>Mean (ng/μl)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iº</td>
<td>13</td>
<td>6,451 (1.74 - 16.64)*</td>
<td>1,765</td>
</tr>
<tr>
<td>IIº</td>
<td>13</td>
<td>3.67</td>
<td>0,505</td>
</tr>
<tr>
<td>IIIº</td>
<td>16</td>
<td>4,902</td>
<td>0,85</td>
</tr>
<tr>
<td>IVº</td>
<td>21</td>
<td>10,809</td>
<td>0,973</td>
</tr>
</tbody>
</table>

* - Bracketed figures give min – max ran

DISCUSSION

In spite of the fact that many research groups are looking for factors engaged in development of colorectal cancer, there is still a need to find a potential marker that may improve the diagnosis
and prognosis of colorectal neoplasm. One such promising factor seems to be the \textit{HMGA1} gene.

In our previous paper (Balcerczak \textit{et al.}, 2003), \textit{HMGA1} gene expression was detected in 63\% of investigated colorectal cancer cases. Our current study produced a slightly lower detection value of 58\%. These data indicate that \textit{HMGA1} gene plays an important role in colorectal carcinogenesis, but is not always required for neoplastic transformation of the cell (mechanism still unknown). The most interesting finding of our previous study was that about 70\% of cases with lymph nodes metastases and all cases (100\%) with diagnosed distant metastases expressed \textit{HMGA1} gene (Balcerczak \textit{et al.}, 2003). Similar to other authors, in our study \textit{HMGA1} gene expression was not observed in normal colon mucosa.

This study has not shown the differences between \textit{HMGA1} gene expression level and patients’ gender, age, and primary tumor localization what confirm our earlier results (Balcerczak \textit{et al.}, 2003). However, lower but not statistically significant, \textit{HMGA1} gene expression level was observed for cancers that had arisen from the rectum. This fact may prove that this gene is not crucial for different mechanisms of right and left part pathogenesis of colon cancer, as suggested by some authors (Thibodeau \textit{et al.}, 1993; Watatani \textit{et al.}, 1996).

\textit{HMGA1} gene expression level was also correlated with several clinicopathological parameters (TNM classification).

We did not find statistically significant differences of \textit{HMGA1} gene expression levels in different T stages. In our previous qualitative analysis (Balcerczak \textit{et al.}, 2003) similar results were achieved in spite of the fact that majority of cases (70\%) belonged to the group with deep wall penetration (T3 and T4), whereas only 30\% belonged to the T1 and T2 groups.

The results obtained earlier haven’t shown any statistically significant correlation between the presence of \textit{HMGA1} gene expression and invasion to lymph nodes (Balcerczak \textit{et al.}, 2003). In the contrary in the present study a significant difference between the levels of \textit{HMGA1} gene expression in cases with and without metastases to lymph nodes was noticed. The higher levels were observed in cases with nodal metastases. \textit{HMGA1} gene transcript was detectable by reverse transcriptase PCR method in all cases with distant metastases and also its level was significantly higher, reached the highest value, in comparison to patients without distant metastases. As a consequence, advanced stages of colon cancers (III, IV) had higher levels of \textit{HMGA1} mRNA. Similar observation was made by Abe and coworkers (Abe \textit{et al.}, 1999) who estimated \textit{HMGA1} by immunohistochemistry.

The higher levels of \textit{HMGA1} gene transcript in cases with lymph node and distant metastases may suggest that its expression is crucial for metastases formation. This hypothesis was confirmed earlier by Wood and coworkers (Wood \textit{et al.}, 2000) who reported that Rat-1a fibroblast cells injected into nude mice, overexpressing \textit{HMGA1} protein form tumors and distant metastases. Also, Reeves and coworkers (Reeves \textit{et al.}, 2001) reported that human breast epithelial cells harboring tetracycline-regulated \textit{HMGA1} transgenes acquire the ability to form both primary and metastatic tumors in nude mice only when the transgenes are actively expressed. Furthermore, expression of either antisense or dominant-negative \textit{HMGA1} constructs inhibits both the rate of proliferation of tumor cells and their ability to grow anchorage independently in soft agar. Additionally, they demonstrated with the use of cDNA array analysis of transcriptional profiles that the \textit{HMGA1} protein modulate the expression of distinctive constellations of genes known to be involved in signal transduction, cell proliferation, tumor initiation, invasion, migration, induction of angiogenesis, and colonization. Recent studies provided interesting and convincing results showing that \textit{HMGA1} gene and its protein product are involved in intra-hepatic metastases formation in humans (Chuma \textit{et al.}, 2004). They conducted comparative gene expression analysis of about 12600 genes by oligonucleotide microarray analysis of hepatocellular carcinoma with and without intrahepatic metastasis. They identified 34 genes, \textit{HMGA1} included, in which expression levels significantly correlated with intrahepatic metastasis. Their further analysis by real-time quantitative reverse transcription polymerase chain reaction and immunohistochemistry confirmed that \textit{HMGA1} gene and its protein product was upregulated and overexpressed in hepatocellular carcinoma with intrahepatic metastasis. Recently Grade \textit{et al.}, 2007 did gene expression profiling of primary colon carcinomas.
in comparison with normal mucosa using oligonucleotide microarrays. *HMGA1* gene was identify as a one among seventeen genes which had above a 5-fold increase. Also Liau et al., 2006 have proven that *HMGA1* silencing resulted in reductions in metastatic potential and tumor growth in vivo. This findings suggest that *HMGA1* may be a novel molecular determinant of invasiveness and metastasis as well as a potential therapeutic target in pancreatic adenocarcinoma.

On the basis of our results (this and previous) it can be suggested that the presence and high level of *HMGA1* gene expression is connected with lymph nodes and especially with distant metastases, thus with poor prognosis for patients. Cases with a lack of detectable nodal and distant metastases but with the presence of *HMGA1* gene transcript, despite its low levels in comparison to those with detectable metastases, should be monitored more closely.

In conclusion, estimation of *HMGA1* gene expression has the potential to be a promising factor which may significantly improve the diagnosis, prognosis, therapy and monitoring of patients suffering from colorectal cancer.

**Acknowledgement**

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**References**


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