Correlation of low expression of hMOF with clinicopathological features of colorectal carcinoma, gastric cancer and renal cell carcinoma

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Abstract. Human MOF (males absent on the first), as a histone acetyltransferase, is responsible for histone H4K16 acetylation in human cells. Recent studies have shown that the abnormal gene expression of hMOF is involved in certain primary cancers. Here, we first report the involvement of hMOF expression in clinically diagnosed primary colorectal carcinoma (CRC) and gastric cancer. Simultaneously, the correlation of hMOF expression and clinicopathological features in CRC, gastric cancer and renal cell carcinoma (RCC) was analyzed. The hMOF mRNA expression was assessed in 44 CRC, 16 gastric cancer and 47 RCC human tissue samples by quantitative PCR (qPCR). Statistical analysis of qPCR data revealed a significant reduction (>2-fold decrease) of hMOF gene expression in CRC, 57% (25/44), 94% (15/16) in gastric cancer and 74% (35/47) in RCC tissues of the patients. In patients with CRC, lymph node metastasis and tumor stage were associated with hMOF expression patterns. However, no significant association between hMOF expression and tumor types emerged (p>0.05). Interestingly, in patients with gastric cancer, although no statistically significant difference was found between adjacent (<2 cm away from the cancer tissue) and normal tissues (>5 cm away from the cancer tissue), >2-fold reduction of hMOF expression in adjacent tissues had already appeared in 35% of patients. In addition, low expression of hMOF was strongly correlated with tumor differentiation (p<0.05) and survival of patients with gastric cancer (p<0.001). While in patients with RCC, downregulation of hMOF was connected to ccRCC and tissues with T1 tumor status. Our results suggest that downregulation of hMOF may be common in cancer tissues, and may represent a novel biomarker for tumor diagnosis.

Introduction

Epigenetic regulation in alteration of chromatin structure is critical for many important cellular processes, including gene transcription, recombination, DNA replication and damage repair (1). Post-translational modifications of the N-terminal tails of histones such as acetylation, methylation, ubiquitilation and phosphorylation are important mechanisms of epigenetic regulation. Current evidence indicates that cancer can occur via disproportionate histone modifications through altering gene expressions including aberrant regulation of oncogenes and/or tumor suppressors (2,3) and unbalanced histone modifications affect genome integrity and/or chromosome segregation (4). In contrast to DNA mutations or deletions, each histone modification process is reversible, thus novel therapies that work by reversing epigenetic effects are being increasingly explored (5).

Histone modification status in cells is dynamically regulated by chromatin modifying enzymes that add and remove covalent modifications to histone proteins. Histone acetylation or methylation as the well-characterized epigenetic modifications is controlled by histone acetyltransferases (HATs) or histone methyltransferases (HMTs) and histone deacetylases (HDAC) or histone demethylases (HDMs). HATs and HMTs can add acetyl and methyl groups, respectively, whereas HDACs and HDMs can remove acetyl or methyl, respectively (6,7). Studies have suggested that epigenetic alterations may play a key role in initiating events in forms of some cancers. Therefore, efforts have been made to understand the role of global changes of epigenetics in the initiation and propagation of various cancers (8,9). For instance, global loss of histone H4K16 acetylation (ac) and histone H4K20 tri-metylation (me3)
as a hallmark of several human cancers have been reported (10). In contrast to this, global changes of other histone modifications seem to be more tumor-specific; for example, low histone H3K4 di-methylation (me2) and H3K9ac levels were shown in breast cancer cells, whereas lung cancer cells show low H3K4me2 but high H3K9ac levels (11,12). Furthermore, global changes of histone modifications also closely associated with clinicopathological factors in some cancers. In patients with renal clear cell carcinoma (RCC), total acetylation levels of histone H3 were inversely correlated with pT-stage, distant metastasis, Fuhrman grading and RCC progression, whereas total histone H4ac deacetylation was correlated with pT-stage and grading (13).

hMOF (hMYST1), a member of the MYST family of HATs, is responsible for histone H4K16ac. Depletion of hMOF in cells leads to genomic instability, reduced transcription of certain genes, defective DNA damage repair and early embryonic lethality (14-16). Biochemical purifications have revealed that hMOF forms at least two distinct multi-protein complexes, MSL and NSL, in mammalian cells. Although the functions of MSL and NSL complexes in human cells are not entirely clear, both complexes can acetylate histone H4K16, suggesting the importance of acetylation of H4K16 in cells (17-19). Abnormal gene expression of the hMOF and its corresponding modification of H4K16 have been found in certain primary cancer tissues. The expression patterns of hMOF in different primary cancers varied. Frequent downregulation of hMOF expression was found in breast cancer, medulloblastoma and RCC (20,21). On the contrary, hMOF was overexpressed in non-small cell lung carcinoma tissues (22,23). In all occasions, hMOF protein expression tightly correlated with acetylation of histone H4K16. Above observations strongly suggest that histone acetyltransferase hMOF and its corresponding histone of H4K16ac might be involved in certain tumorigenic pathways.

With aging and urbanization progresses, the number of cancer patients has increased dramatically. Colorectal cancer (CRC) has become one of the major causes of mortality and morbidity, and is the third most common cancer in men and the second most common cancer in women worldwide (24). RCC is one of the most common genitourinary malignancies, accounting for 3% of all cancer worldwide (25). However, gastric cancer, another common tumor, is the second most common cause of cancer deaths in the world (26). In spite of recent studies indicating that global histone modification, such as methylation of H3K4 and H3K27, correlate with the outcomes in patients with above-mentioned cancers (27,28), there have so far been no reports on HAT hMOF and its corresponding modification in CRC and gastric cancer. Here we first examined the hMOF mRNA and protein expression levels in primary CRC and gastric cancer by quantitative PCR (qPCR) and western blotting (WB). In addition, we recently reported the hMOF HAT is frequently downregulated in ovarian cancer and RCC (29,21), here we further analyzed the relationship between low-expression of hMOF and clinicopathological features in RCC.

Materials and methods

**Tissue collection.** Human tumor tissues (CRC, gastric cancer and RCC) and normal tissues were collected from patients with primary CRC cases between July 2012 and March 2013, from patients with primary stomach cancers between September 2008 and August 2011, and from patients with primary RCC cases between May 2009 and May 2012. Cancer patients underwent radical tumor surgery at the First Clinical Hospital of Jilin University. Written informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Board of School of Medicine, Jilin University. Patient medical records including patient age and gender, tumor staging, pathological diagnosis, and surgical records were reviewed. Tumors were staged according to the 2010 TNM classification system using the American Joint Committee on Cancer (AJCC) stage grouping (30). None of the patients received chemotherapy or radiotherapy before surgery.

**Antibodies.** Anti-H4K16ac (H9164) polyclonal antibody was purchased from Sigma. Anti-GAPDH and anti-hMOF were raised against bacterially expressed proteins (Jilin University).

**Reverse transcription PCR (RT-PCR).** Total RNA from tumor (CRC, gastric cancer, ovarian cancer or RCC) or normal tissues were isolated using TRIzol® LS Reagent (Invitrogen, CA, USA). Then, 1 µg of RNA from each sample was used as a template to produce cDNA with PrimeScript 1st Strand cDNA Synthesis kit (Takara). hMOF and GAPDH mRNA levels were analyzed by quantitative PCR with an Eco Real-Time PCR System (Illumina). All PCR reactions were finished as follows: initial denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Primer sets used for PCR were as follows: GAPDH, 5'-ATCACTGCCACCAGGAGAAC-3' (forward) and 5'-ATGAGGTCCACCACCCTGTT-3' (reverse), yielding a 460-bp product; hMOF, 5'-GGCTGCTGGCTCTGTACGTGGACGAGTGGGTAGACAA-3' (forward) and 5'-TGGTGCAATCCCTGAGGGCTCTCTTTTAA-3' (reverse) and 5'-TGTTGATCGCCCTCATGCTCCTT-3' (reverse), yielding a 227-bp product.

**Western blotting (WB).** Cancer tissue (200 mg) or normal tissue samples were homogenized with liquid nitrogen and solubilized in 200 µl cold PBS containing 1.0% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 0.05 mM PMSF and protease inhibitor cocktail. The homogenate was swirled and kept on ice for 30 min. Whole-cell extracts were sonicated (Scientz-ID, China) for 10 sec with 50% duty cycle and centrifugation (13,000 x g for 30 min). The protein concentration of the resulting supernatant was measured using the Bio-Rad Protein Assay kit (500-2021). Equal amounts of protein from tissue whole-cell lysates were mixed with 4X SDS-containing sample buffer and boiled for 5 min at 95°C. Denatured proteins were then separated by 12% SDS-PAGE. Specific proteins were detected by WB using hMOF and GAPDH polyclonal antibodies.

**Immunohistochemical staining (IHC).** Formalin-fixed and paraffin-embedded CRC tissue blocks were supplied from The First Clinical Hospital of Jilin University. IHC was performed essentially as described (31,32). Anti-hMOF and acetylated H4K16 polyclonal antibodies (H9164) were used at a 1:500 dilution.
Statistical analysis. The western blot images were scanned and quantified by Quantity One Basic software (Bio-Rad). Differences in gene and protein expression between tumor and normal tissues were statistically analyzed using SPSS 17.0 (SPSS, Inc., Chicago IL, USA). Statistical comparisons were analyzed using the Student’s t-test. Values of p<0.05 were considered to be statistically significant.

Results

Reduction of hMOF gene expression level is observed in CRC. To investigate the involvement of hMOF gene expression in the pathogenesis of primary CRC, we first measured hMOF mRNA using qPCR in 44 patients diagnosed with CRC. Compared to matched normal tissues, the gene expression of hMOF was significantly decreased in CRC tissues (p<0.05; n=44) (Fig. 1B). As shown in Fig. 1A, analysis of performed mRNA expression of 44 samples revealed significant (>2-fold decreased) downregulation of hMOF mRNA in 57% (25/44) of patients, whereas 7% (3/44) of patients showed significant (≥2-fold increased) upregulation of hMOF. The relationships between hMOF expression and clinical parameters, including age and gender, tumor types, histological grade, and lymph node metastasis were further analyzed. A significant downregulation of hMOF was only observed in male (p<0.05), patients >60 years of age (p<0.05) or patients with lymph node metastasis (p<0.05) (Fig. 1B). In addition, obvious reduction of hMOF gene expression was also detected in tissue with a T4 tumor status (p<0.05) (Fig. 2A), but there was no association between low-expression of hMOF and histological tumor types of CRC (Fig. 2B).

hMOF gene expression in gastric cancer. Next, to understand the implication of hMOF gene expression in the pathogenesis of primary gastric cancer, 16 clinical gastric cancer tissues and matched adjacent (<2 cm away from the tumor) and/or normal (>5 cm away from the tumor) tissues were used. As shown in Fig. 3, compared to matched normal tissues, hMOF mRNA expression was significantly downregulated (>2-fold decrease) in 94% (15/16) of tumor tissue samples. Interestingly, hMOF
expression in adjacent tissues also showed great reduction (>2-fold decrease) in 35% of samples. The summarization of the clinical characteristics including age and gender, cell differentiation and survival of patients is shown in Table I. Decreased hMOF expression was observed in >70 years of age patients (p<0.01) and in the female group (p<0.01), whereas, downregulation of hMOF expression was strongly associated with poorly-differentiated tumor tissue (p<0.01). In addition, significant connection was observed between hMOF expression and <1-year survival of patients (p<0.001). In contrast, no statistically significant difference was found between adjacent and normal tissues.

Correlation of hMOF expression with clinicopathological characteristics of renal cell carcinoma. We previously found downregulation of hMOF (91% of patients, 19/21) in RCC, resulting in decreased hMOF protein, and this reduction is tightly correlated with histone H4K16 acetylation (21). To further confirm our results and to evaluate the relationship between hMOF expression and clinicopathological parameters, we measured hMOF mRNA using qPCR in 47 patients diagnosed with RCC. Analysis of qPCR data revealed a significant (>2-fold decreased) downregulation of hMOF mRNA in 74% (35/47) of patients, whereas only 6% (3/47) of patients showed significant (>2-fold increased) upregulation of hMOF (Fig. 4A). These results strongly support our previous data. As shown in Fig. 4B, low-expression of hMOF was found in both >60 years of age (p<0.05) and <60 years of age (p<0.05) groups, statistically significant reduction of hMOF was recorded only in male patients. In addition, there was a correlation between low-expression of hMOF and ccRCC (p<0.05) or tissue with T1 tumor status (p<0.05). However, declined hMOF expression showed no association with the RCC tissue Fuhrman grading (data not shown).

Reduction of hMOF protein levels in primary human CRC. To determine whether the reduction of hMOF gene expression
resulted in reduced hMOF protein levels, western blotting and IHC staining approaches were used. Aliquots of whole cell extract from 22 paired, initially selected CRC and matched normal tissues were analyzed by western blotting with hMOF antibodies (GAPDH as internal reference protein). As expected, frequent reduction of hMOF protein in CRC compared to matched normal tissues was observed. Western blot analysis revealed that hMOF protein levels were significantly lower in CRC tissues compared to matched normal tissues. The reduction in hMOF protein was consistent across all tumor samples analyzed.

Figure 3. Expression patterns of hMOF mRNA in gastric cancer. Total RNA was isolated from 16 clinically diagnosed gastric cancer tissues and its matched adjacent (<2 cm away from the tumor tissue, and cell morphology was normal under the microscope) and normal tissues (>5 cm away from the tumor) (bottom). Each bar is the log2 value of the ratio of hMOF expression levels between CRC and matched normal tissues or adjacent and normal tissues from the same patients (top).

Table I. Relationship between hMOF gene expression (qPCR) and clinicopathological characteristics of gastric cancer.

<table>
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<tr>
<th>Factor</th>
<th>Case (n)</th>
<th>Normal Mean ± SD</th>
<th>Adjacent Mean ± SD</th>
<th>Cancer Mean ± SD</th>
<th>p-value Nor vs. Adj</th>
<th>p-value Nor vs. Can</th>
<th>p-value Adj vs. Can</th>
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<tr>
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<td>Age (years)</td>
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<tr>
<td>≤70</td>
<td>10</td>
<td>28.6±10.1</td>
<td>5.57±5.17</td>
<td>4.27±6.46</td>
<td>0.291</td>
<td>0.267</td>
<td>0.708</td>
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<tr>
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<td>6</td>
<td>8.38±4.58</td>
<td>12.5±12.4</td>
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<td>0.338</td>
<td>0.00172‡</td>
<td>0.0224‡</td>
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<td>8</td>
<td>8.39±4.85</td>
<td>9.02±6.01</td>
<td>2.52±2.16</td>
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<td>0.00737‡</td>
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<tr>
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<td>7.18±6.42</td>
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<td>0.902</td>
<td>0.00148‡</td>
<td>0.0215‡</td>
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<td>Survival of patients</td>
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<td>&gt;12 months</td>
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<td>9.72±6.25</td>
<td>4.89±5.35</td>
<td>0.336</td>
<td>0.211</td>
<td>0.119</td>
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<tr>
<td>&lt;12 months</td>
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<td>4.99±2.72</td>
<td>1.01±0.66</td>
<td>0.541</td>
<td>0.000581‡</td>
<td>0.00124§</td>
</tr>
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</table>

‡p<0.05; †p<0.01; §p<0.001.
Figure 4. Correlation of hMOF expression with clinicopathological characteristics of RCC. (A) Frequent downregulation of hMOF gene observed in RCC. Total RNA was isolated from 47 clinical RCC tissues and its matched normal tissues. Y-axis exhibits the log2 value of the ratio of hMOF expression levels between RCC and normal tissues. (B) Relationship between low-expression of hMOF gene and clinicopathological features of RCC. mRNA expression levels of hMOF from 47 RCC tissues were compared to matched normal tissue. qPCR results were analyzed between ages, gender, tumor types and pathologic stage. Significant difference, *p<0.05 and **p<0.01.

Figure 5. Reduction of the hMOF protein levels were detected in selected CRC tissues. Whole cell extract was prepared from tissues and equivalent total protein amount of whole cell extract was subjected to SDS-PAGE in 12% gels, and proteins were detected by western blotting with anti-hMOF and GAPDH antibodies (bottom). Western blot images were quantified by Quantity One software (Bio-Rad) and normalized by GAPDH levels. Significant difference, *p<0.05.
matched normal tissues was detected (Fig. 5, bottom panel). The quantified protein levels (Quantity One software) were analyzed by t-test. As shown in Fig. 5 (upper panel) hMOF protein expression levels were significantly reduced in CRC tissues (p<0.05). To determine whether the expression of hMOF was tightly correlated with histone H4K16 acetylation, we performed immunohistochemical staining (IHC) for hMOF and histone H4K16 acetylation in the formalin-fixed paraffin-embedded tissue sections of selected CRC patients. The results revealed that hMOF protein expression was correlated with acetylation of histone H4K16 in parallel (Fig. 6).

**Discussion**

Histone acetylation status in cells is controlled by HATs and HDACs. Any factor that creates an imbalance can lead to abnormal cell function, even cancer. The HAT hMOF belongs to the MYST (Moz-Ybf2/Sas3-Sas2-Tip60) family, and is believed to be responsible for histone H4K16 acetylation in both Drosophila and human cells (33,18,34). It is known that hMOF participates in many biological processes, including gene transcription, cell proliferation, differentiation, DNA repair response (14,16,17,19,33). Although little is known about the mechanism of hMOF in tumor development and progress, the expression of hMOF in limited types of clinical cancer tissues has been reported by several research groups. Frequent downregulation of hMOF in primary breast carcinomas, renal cell carcinoma and medulloblastomas was found, and the reduction of hMOF protein expression tightly correlated with acetylation of H4K16 in those tumors (20,21). In contrast, the expression of hMOF in non-small cell lung carcinoma tissues was frequently elevated (22,23).

In this study, we first investigated the expression of HAT hMOF in primary CRC and gastric cancer tissues by qRT-PCR. The results revealed that either hMOF mRNA expression or hMOF protein expression was downregulated in human primary CRC (Figs. 1 and 5), and hMOF protein expression was correlated with acetylation of histone H4K16 in parallel (Fig. 6). Further analysis of qPCR data revealed that expression of hMOF was associated with the age of the patients, gender, lymph node metastasis, and tissues with a T4 tumor status. It is noteworthy that the abnormal expression of hMOF had already occurred in the adjacent tissues (<2 cm away from the cancer tissue). Compared to matched normal tissues, although no statistically significant difference was found between adjacent and normal tissues, a significant reduction (>2-fold decrease) of hMOF expression in adjacent tissues had already appeared in 35% of patients with gastric cancer. In addition, low-expression of hMOF in gastric cancer was strongly associated with poorly-differentiated tumor tissue.

![Figure 6. Immunohistochemical staining for hMOF and histone H4K16ac in CRC tissues. (A) Hematoxylin and eosin staining (H&E), magnification x200. (a) Well-differentiated colorectal carcinoma; (b) moderately-differentiated colorectal carcinoma; (c) poorly-differentiated colorectal carcinoma. (B) (a and d) Normal rectum tissue; (b and e) hMOF or H4K16ac-positive; (c and f) hMOF or H4K16ac-negative.](image-url)
and <1-year survival of patients (Table I). Our results demonstrate that abnormal expression of hMOF might be involved in tumorigenesis of CRC and gastric cancer. In line with the results obtained from a previous study (21), a significant (> 2-fold decreased) downregulation of hMOF mRNA in 74% (35/47) of patients with RCC was observed. Downregulation of hMOF was strongly associated with ccRCC and with T1 tumor status. This suggests that abnormal expression of hMOF is not only associated with tumor types, but might be also an early biomarker in RCC.

In conclusion, downregulation of hMOF was observed in CRC, gastric cancer and RCC tissues. Although larger series of clinical cases and analyses of overall survival are needed, the molecular mechanism linking loss of hMOF expression may be involved in the above described cancer progression.

Acknowledgements

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