Augmented Membrane Type 1 Matrix Metalloproteinase (MT1-MMP):MMP-2 Messenger RNA Ratio in Gastric Carcinomas with Poor Prognosis

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ABSTRACT

The activation of zymogen and the amount of proteinase and its inhibition are important in determining the eventual activity of matrix-degrading enzymes involved in tumor aggressiveness. To evaluate a gene complement leading to matrix metalloproteinase 2 (MMP-2; M, 72,000 gellatinase) activity, membrane type 1 MMP (MT1-MMP), urokinase-type plasminogen activator, MMP-2, and tissue inhibitor of metalloproteinase 2 transcriptional levels were measured in gastric carcinoma biopsies. Comparative tumor:normal tissue reverse transcription-PCR in a cohort of 25 patients revealed up to a 10-fold difference in the expression of MT1-MMP, a metalloproteinase that has been proposed as a membrane receptor activator of MMP-2; a 1-unit increment resulted in a 30% risk to survival. A 20% risk also resulted from a 1-unit increment in the MT1-MMP:MMP-2 ratio, which showed differences of up to 15-fold. Instead, the expression of urokinase-type plasminogen activator, which trips off a cascade ending in the activation of MMP-2, as well as the expression of MMP-2 itself and its inhibitor, tissue inhibitor of metalloproteinase 2, lacked correlation with patient follow-up. Zymography revealed MMP-2 activities that were often in conflict with the transcription results and also with follow-up. The results suggest the evaluation of MT1-MMP and/or MT1-MMP:MMP-2 transcription as a new preoperative molecular-level prognostic factor for gastric carcinoma.

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INTRODUCTION

MMP-2, also known as gelatinase A or M, 72,000 gelatinase, is active against gelatin and basement membrane components (type IV collagen and laminin; Refs. 1 and 2) and has been repeatedly shown to play a crucial role in the invasive metastatic phenotype. As with other enzymes, MMP-2 activity results from the balance between positive and negative regulation (3); excessive proteolysis may result from an increased expression of proteinase or activation of the zymogen form and/or insufficient local inhibition of lytic activity.

Overproduction of MMP-2 or underproduction of its tissue inhibitor, TIMP-2 (4), may result in matrix degradation crucial for metastasis. Immunohistochemistry and in situ hybridization in human gastrointestinal neoplasias have shown that these carcinomas contain enhanced amounts of MMP-2 (5). Immunohistochemistry showed that augmented MMP-2 levels in the serum of lung (6), breast (7), and gastric (8) carcinoma patients correlate with cancer aggressiveness, and zymography in gastric carcinoma tissue showed a correlation with overall survival (9). However, conflicting results were registered in these tumors by both techniques (10, 11).

RT-PCR analysis may offer a more in-depth approach: a lower ratio of MMP-2:TIMP-2 mRNA has been found in lymph node-negative breast carcinoma biopsies than in lymph node-positive samples (12) and in mouse mammary carcinomas treated with an antimetastatic drug than in untreated cases (13). This implies that the increased expression of MMP-2 in more aggressive cancer tissues may overcome the strict regulation exerted by its inhibitor. The enzyme:inhibitor mRNA ratio may thus be an early indicator of aggressiveness and may be more reliable than the enzyme alone.

Nevertheless, an additional key determinant of invasive behavior is pro-MMP-2 activation. Overproduction of proenzyme and the generation of proteolytic activity are not equivalent, and the close control of activation is still an open field. Two major candidates have been proposed to date as gelatinase activators: (a) MT1-MMP (14); and (b) uPA/plasmin cascade (15). Whereas each of these can exert independent proteolytic activity, both may trigger cell invasion through the activation of progelatinases on the tumor cell surface.

Some investigations link the expression of MT1-MMP (16, 17), uPA (18–20), MMP-2 (5–9, 12, 13, 21), or TIMP-2 (12, 13, 21, 22), separately or in combination, to invasive and metastatic...
aggressiveness, but no studies have yet focused on the expression of this entire gene complement involved in the eventual MMP-2 activity.

Gastric carcinoma is one of the most aggressive tumors, and patients face a poor prognosis unless it is detected and cured in an early stage (5-year survival, 20–25% after curative surgery). Early knowledge of the parameters linked to invasive behavior would prove useful in assessing a patient’s eligibility for neoadjuvant chemotherapy as well as in postoperative treatment.

Therefore, the present investigation aims to investigate by RT-PCR in a cohort of biopsies from human aggressive tumors such as gastric carcinoma whether the balance between mRNAs for MMP-2, its inhibitor TIMP-2, and two potential activators, MT1-MMP and uPA, may be of prognostic value in cancer disease and to compare the PCR results with zymography.

**MATERIALS AND METHODS**

**Biopsy Specimens.** Biopsy specimens from 25 patients (11 women and 14 men) with gastric carcinoma were obtained immediately after resection from the 2nd Surgical Department of Padova University Hospital (Padova, Italy). Specimens (50–100 mg) of tumor without necrotic lesions and of corresponding normal adjacent tissue were snap-frozen in liquid nitrogen and transferred to a –80°C freezer until RNA extraction (see below). Stage and follow-up information were obtained and updated (Table 1) only on completion of RT-PCR analyses. Survival periods ranged between 2–24 and 19–86 months for the 12 deceased and 13 living patients, respectively. Three patients underwent postoperative chemotherapy, and no relapses were revealed in the living cohort.

**RNA Extraction and RT-PCR.** RT-PCR was used to quantitate the MMP-2:TIMP-2, MT1-MMP:MMP-2, and uPA:MMP-2 ratios according to specific methods described by Onisto et al. (23). Total RNA was extracted from tumor tissues using the RNAzol method (Ultraspec RNA; Biotecx). Tissues were snap-frozen in liquid nitrogen, rapidly mortar-ground, suspen- sed in Ultraspec solution, and then treated as described previously (23). RNA yield and purity were checked by spectrophotometric determinations at 260 and 280 nm.

Reverse transcription of 5 µg of RNA was carried out as described previously (23). The 10−2 dilutions were then divided into aliquots in amplification tubes and stored at −20°C until PCR. A typical PCR reaction mixture was prepared as follows: (a) equal volumes of Taq DNA polymerase (5000 units/ml; Pharmacia) and TaqStart antibody (1.1 µg/µl; Clontech) were preincubated in 4 volumes of kit dilution buffer for 10 min at room temperature; and (b) 2.4 µl of this mixture were added to 5 µl of 10X reaction buffer (Pharmacia), 1 µl of deoxynucleotide triphosphate (10 µM each), 2 µl of primers (5 µM each) as listed in Table 2, and 20 µl of cDNA dilution and water to a 50-µl final volume. Amplification was performed in sequential cycles including a 1-min denaturation at 94°C, annealing con-
cycles using fixed reaction conditions (see above). The specific experiments were performed in three random specimens by blotting onto a nylon membrane (Hybond N+), again verified for each target molecule in high-scoring samples. The nylon membrane was soaked for 5 min in neutralizing solution and cross-linked by exposure to UV. Specific DNA probes were synthesized according to the published sequence (14). Probes were labeled with [α-32P]dCTP (Amersham) by means of alkali blotting (0.6 M NaCl and 0.4 M NaOH). After the transfer, the nylon membrane was soaked for 5 min in neutralizing solution and cross-linked by exposure to UV. Specific DNA probes for MMP-2, TIMP-2, and uPA were obtained as described previously (22); probes for MT1-MMP were synthesized according to the published sequence (14). Probes were labeled with [α-32P]dCTP (Amersham) using an oligolabeling kit (Pharmacia Biotech) and then hybridized to the membrane, and the autoradiograms were analyzed by a scanning densitometer (GS300 Hoefer). The linear range of amplification was measured three times; in cases in which the SD of the triplicate bands exceeded 15%, PCR amplification or Southern blot was repeated. Data of triplicate bands were processed to again verify the average of three densitometric scans of the bands shown at the top.

For each target molecule, preliminary linear range-finding experiments were performed in three random specimens by PCR-amplifying cDNA aliquots for an increasing number of cycles using fixed reaction conditions (see above). The specific number of cycles was then determined, as reported in Table 2. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands. After amplification, triplicate 20-μl aliquots were electrophoresed as a control to identify progelatinolytic and active digestion bands.

**Table 2** Oligonucleotide sequences and conditions for PCR

<table>
<thead>
<tr>
<th></th>
<th>MMP-2 (Ref. 31)</th>
<th>TIMP-2 (Ref. 4)</th>
<th>MT1-MMP (Ref. 14)</th>
<th>uPA (Ref. 18)</th>
<th>GAPDH (Ref. 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>5'-ACCTGAGATGCCTGTCGGAC-3'</td>
<td>5'-TGCGAGTGCTCTCCCCCGTGAC-3'</td>
<td>5'-CAAGCAACTTATGGGAGTGC-3'</td>
<td>5'-GTGCTTACCTTTAGGTT-3'</td>
<td>5'-ACCAGCTTCCATGC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-TGTTGCAAGACAGGCGCAG-3'</td>
<td>5'-TTATGGGTCTCTGATGCGA-3'</td>
<td>5'-GTTCTACCTTTAGGTT-3'</td>
<td>5'-CCAUTCCTCCCTTGTTGACTG-3'</td>
<td>5'-TTATGGGTCTCTGATGCGA-3'</td>
</tr>
<tr>
<td>Dimension</td>
<td>447 bp</td>
<td>647 bp</td>
<td>654 bp</td>
<td>564 bp</td>
<td>600 bp</td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C for 30 s</td>
<td>62°C for 60 s</td>
<td>62°C for 30 s</td>
<td>62°C for 30 s</td>
<td>62°C for 30 s</td>
</tr>
<tr>
<td>amplification</td>
<td>28 cycles</td>
<td>27 cycles</td>
<td>28 cycles</td>
<td>28 cycles</td>
<td>25 cycles</td>
</tr>
</tbody>
</table>

The MMP-2:TIMP-2, MT1-MMP:MMP-2, and uPA:GAPDH ratios (tumor versus normal) were also calculated; i.e.,

\[
\frac{\text{MMP-2}_{\text{tumor}}}{\text{MMP-2}_{\text{normal}}} = \frac{\text{TIMP-2}_{\text{tumor}}}{\text{TIMP-2}_{\text{normal}}}
\]

Only on completion of densitometric and mathematical analyses were tumor stage and survival obtained from the Pathology Department (Table 1). Patients were then separated into living and deceased groups. To evaluate which variable (sex, stage, or molecular markers) may affect survival, Cox’s proportional hazard model was applied using the SPSS statistical package (SPSS, Inc., Chicago, IL; Ref. 24). For survival-affecting variables, overall survival curves were estimated accordingly.

**Zymographic Analysis.** Gelatinolytic activity in tissue biopsies was assayed as described previously (11). Part of the tissue ground on a mortar in liquid nitrogen was weighed and suspended in SDS-PAGE sample buffer. Without heating the samples, zymography was carried out by electrophoresing 1 mg of tissue in a 9% polyacrylamide/0.1% gelatin gel in the presence of SDS. After electrophoresis, the gels were washed twice for 15 min with 2.5% Triton X-100, incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl and 5 mM CaCl2, stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250, and then destained in the same solution without dye; clear bands represent areas of gelatinolysis on the blue background. Culture medium conditioned by HT1080 melanoma cells was coelectrophoresed as a control to identify progelatinolytic and active gelatinolytic bands (25).

Digestion bands were analyzed by a simple visual compar-
ison of tumor and normal samples, and variations over normal tissue were registered (data not shown).

RESULTS

Assay Validation. The chosen primers were appropriate and generated specific single amplicons with no additional bands when analyzed by electrophoresis. To verify the appropriate conditions for amplicon quantitation, initial linear range-finding experiments were performed for each marker, amplifying fixed DNA dilutions (10^{-2}) for an increasing number of cycles. The amplification reactions were linear between the following number of cycles: (a) 26–32 for MMP-2; (b) 24–30 for TIMP-2; (c) 22–32 for MT1-MMP; (d) 26–34 for uPA; and (e) 22–28 for GAPDH. An example of the relationship between the number of amplification cycles and the amplicon yield is shown in Fig. 1 for MMP-2.

RT-PCR was then routinely performed on 10^{-2} cDNA dilutions, amplifying each marker for the linear range-fitting number of cycles, as reported in Table 2. This produced a sufficient signal for densitometric analysis, except for MT1-MMP in five cases in which no detectable amplicons were obtained in either normal or tumor tissue (Table 1). The linear range of amplification was verified for high-scoring samples, and no case revealed an underestimation attributable to plateau-approaching reactions (data not shown). Fig. 2 shows an example of the amplification products for MMP-2, TIMP-2, MT1-MMP, uPA, and GAPDH in normal (N) and carcinoma (T) gastric specimens of patient 25 (S. G.).

Separate MMP-2, TIMP-2, MT1-MMP, and uPA Variations in Normal and Tumor Tissues. mRNA levels for MMP-2, TIMP-2, MT1-MMP, and uPA normalized on the basis of control gene expression (GAPDH) were found to be either augmented or reduced in tumor tissue in comparison with the normal counterpart (Table 1); in different patients, tumor: normal MMP-2 ranged between approximately 0.1 and 14-fold, TIMP-2 ranged between one-fifteenth and 12-fold, MT1-MMP ranged between 0.03 and 10-fold, and uPA ranged between 0.1 and almost 6-fold. The MT1-MMP value is not reported (Table 1, ND) for two living and three deceased patients due to undetectable amplicons in both normal and tumor tissue for the standard number of PCR cycles.

MT1-MMP mRNA in tumor tissue registered below that in normal tissue (tumor:normal < 1) in only 1 of 9 deceased patient biopsies with detectable markers but in 5 of 11 biopsies from patients in the living group (Fig. 3). The highest tumor: normal MT1-MMP values were 10.2 (deceased group) and 4.9 (living group).

According to Cox's regression model, only MT1-MMP affects survival: an increase of 1 unit of MT1-MMP (i.e., a doubling of MT1-MMP mRNA expression in tumor versus normal tissue) corresponds to a 30% risk increase with \( P = 0.0437 \) (Fig. 4). Cox's model shows survival to be independent of MMP-2, TIMP-2, and uPA transcript levels; patient gender; and stage (from IA to IV) of the tumor.

Determination of MMP-2:TIMP-2, MT1-MMP: MMP-2, and uPA:MMP-2 Ratios. These balances (transcripts in transformed versus normal specimens of human gastric tissues) can be calculated from the values in Table 1 and are as follows: (a) MMP-2:TIMP-2 registered from approximately 0.1 to over 6-fold; (b) MT1-MMP:MMP-2 registered from 0.02 to 15-fold; and (c) uPA:MMP-2 registered from 0.1 to 4-fold.

The highest increase in tumor:normal MT1-MMP:MMP-2 (15-fold) was registered in a stage IV patient (patient 23) who survived for 3 months after surgery despite a 30% decrease in the expression of MMP-2 and a 60% increase in TIMP-2. In contrast, in the living group, MT1-MMP:MMP-2 mRNA in tumor tissue registered below that of normal tissue in 7 of 11 patient biopsies with detectable markers, with the highest values around 2-fold in 3 cases.

According to Cox's regression model, only MT1-MMP: MMP-2 affects survival: a 1-unit increment in MT1-MMP: MMP-2 corresponds to a 20% risk increase with \( P = 0.0175 \) (Fig. 4). Survival is not affected by MMP-2:TIMP-2 or uPA: MMP-2.
Patients.

MT1-MMP and MT1-MMP:MMP-2 mRNAs in gastric carcinoma patients. Regarding the activated form in the tumor, a relative increase of MMP-2 was registered in six living and seven deceased patients, in tumor tissue than it was in normal tissue (Fig. 5). The trend (Fig. 5, patient 11), four living and six deceased patients oppose the trend (Fig. 5, patient 17); and (c) in two deceased patients, both with increased mRNA expression, pro-MMP-2 was lower in tumor tissue than it was in normal tissue (Fig. 5, patient 25). Regarding the activated form in the tumor, a relative increase of MMP-2 was registered in six living and seven deceased patients, as expected from the corresponding augmented MT1-MMP and/or uPA mRNAs (patients 11 and 25), and also in three living and three deceased patients with decreased expression of activators (patient 17). Zymography could not be performed in four cases (patients 1, 2, 6, and 24) due to paucity of the specimen.

**DISCUSSION**

Comparative RT-PCR (tumor versus normal tissue) on small gastric carcinoma biopsies focusing on the cascade(s) leading to active MMP-2 shows that one of the four markers analyzed, MT1-MMP (potential membrane activator of MMP-2; Ref. 14), is significantly elevated in the more aggressive tumors. The introduction of RT-PCR for the detection of tissue-specific transcripts improved sensitivity to the detection of 1 cancer cell in 10^7 normal cells, enabling molecular biological analyses of small samples such as biopsy specimens, although analysts must be aware of reproducibility and representativity (26). Although the evaluation of TIMP-1 in small gastric carcinoma samples has been shown to reflect the whole tissue under investigation (27), the problem of representativity must always be considered, especially in preoperative sampling and in setting up the experiments. In addition, the proximity of normal tissue to the carcinoma sample must be taken into account, because this tissue may contain fibroblasts, macrophages, and so forth that are under the influence of activating factors from the tumor. Preliminary amplification linear range-finding experiments are also essential for achieving confident results. With these precautions, a number of markers, including cytokeratin 19 (26), mdr-1 (28), MMP-2, MMP-9, TIMP-1, and TIMP-2 (9, 12, 13, 27), have already been evaluated by semi-quantitative comparative (tumor:normal) RT-PCR. Using the same approach, the optimal number of PCR cycles was identified for specifically amplifying four molecules involved in the eventual degradation of basement membrane components in the linear range of reaction: (a) MMP-2; (b) TIMP-2; (c) MT1-MMP; and (d) uPA. In addition, triplicate amplifications gave evidence of the reproducibility of the results.

RT-PCR analysis now reveals that MT1-MMP, together with the MT1-MMP:MMP-2 ratio, is a factor linked to overall survival. Although the risk P for an increment of the former shows moderate significance (a larger sample size would have been desirable), the value for the latter allows more confidence; a 1-unit increment of MT1-MMP and MT1-MMP:MMP-2 expression in tumor tissue versus normal counterpart tissue results in a 30 and 20% risk increase, respectively. The likely explanation is that raised MT1-MMP levels lead to more MMP-2 activation and consequently to more aggressive behavior. Even so, high expression of MT1-MMP does not necessarily parallel short survival, and our data do indeed show exceptions; i.e., in the cohort of deceased patients, patient 14 had the longest survival time (24 months), despite being at stage IV and having an almost doubled tumor:normal MT1-MMP mRNA ratio, but little MMP-2 was expressed in this case.

Total MMP-2 was generally found by zymography to be more abundant in tumor tissues than in normal tissues (examples are shown in Fig. 5), with most cases showing a prevalence of zymogen over the activated form, which is in line with a previous report (9). Nevertheless, in some cases, this is appar-
One Unit = 30% risk (p = 0.0437)

One Unit = 20% risk (p = 0.0175)

Fig. 4 Survival curves for MT1-MMP and MT1-MMP:MMP-2 transcripts in gastric carcinomas as determined by Cox’s proportional hazard model. Lines represent the cumulative survival for levels (tumor:normal) of MT1-MMP and MT1-MMP:MMP-2 corresponding to mean (2.676 and 2.447, respectively) 0.5 and 5.

Fig. 5 Gelatin zymography (9% SDS-PAGE) of 1 mg of normal (N) and carcinoma (T) gastric tissue. Gelatin was digested by the zymogen (upper band; Mn, 72,000) and the activated form (lower band) of MMP-2. Numbers at the bottom refer to patient numbers as listed in Table 1.

In contrast with the present RT-PCR results, showing lower MMP-2 mRNA levels in the tumor samples than in the normal counterpart or vice versa. The discrepancy might have its roots in the extracellular matrix representing a storage compartment for some proteinases to which peripheral stromal cells may also contribute (29). In this case, an accumulation of enzyme would not necessarily be in contrast with a low level of mRNA if some feedback mechanism shuts off messenger expression after the secretion and/or binding of protein. The capacity of tumor cells to bind MMP-2 at the membrane level would then be more significant than the amount of enzyme itself. This is in line with a recent report by Mazzieri et al. (15) demonstrating activation of progelatinases by the urokinase/plasmin cascade only when uPA is bound to its membrane receptor. Whether or not the same consideration is applicable to the receptor activator MT1-MMP or whether a concerted action occurs between MT1-MMP and uPA in pro-MMP-2 activation remains to be elucidated.
The relevance of membrane binding is also supported by the fact that in the living group, those patients with no MT1-MMP or only traces of MT1-MMP had a good follow-up, despite a good or strong increase in MMP-2 in the tumor as revealed by zymography (patients 3, 5, and 7). The two MT1-MMP-negative patients with long survival times in the deceased group, patients 15 and 16, also give striking support to the crucial role played by membrane binding.

Deeper knowledge of MT1-MMP function toward MMP-2 is needed to explain why the MT1-MMP increase (PCR) was paralleled only in some cases by augmented levels of activated MMP-2 (zymography). Certainly, RT-PCR evaluation of MMP-2 alone was not informative regarding survival in this small group of gastric carcinoma patients, whereas in more numerous cohorts, both zymography (9) and the counting of positive cells by immunohistochemistry revealed a significant correlation between MMP-2 and follow-up (21).

The MMP-2:TIMP-2 mRNA balance lacks correlation with either stage or follow-up in the present study on gastric carcinoma, whereas the same balance was found to be increased in lymph node-positive patients compared with lymph node-negative patients in a previous study of breast carcinoma (12). So how crucial is the regulation exerted by the TIMP-2 inhibitor in the control of invasion, and in what phase? At the same time, it must be recalled that gastric and mammary carcinomas differ significantly in their aggressive behavior; the postoperative 5-year survival rate is almost twice as much in the latter. Certainly, the relative lack of early symptoms in gastric cancer compared with breast cancer plays an important role in delayed diagnosis (and survival), and differences in the molecular pattern may then be attributable to the progression phase and/or the different type of cancer.

In addition, uPA mRNA expression does not correlate with aggressive phenotype in this study. The cell-bound uPA/plasmin cascade has the potential to activate both pro-MMP-2 and pro-MMP-9 directly, without metalloproteinase intermediates (15); uPA could then play a crucial role in tumor invasive behavior, and an increase in the uPA level may be expected in aggressive tumors. Nevertheless, conflicting reports show a correlation in breast, gastric, and other cancers between uPA expression and tumor invasion (18, 19) and also between the suppression of metastatic potential and up-regulation of uPA in Lewis lung carcinoma after pharmacological treatment (22).

Although we now register levels of uPA expression varying from patient to patient, uPA mRNA is always expressed in tumor tissue, albeit weakly in a few cases. How efficiently this messenger is translated into protein remains to be determined. It may be suggested that even a reduced amount of uPA contributes sufficient activation of plasminogen, which is ubiquitous in tissue fluids, given the presence of uPA-R. uPA-Rs are in fact essential to the occurrence of the proteolytic cascade; other investigations report that high uPA-R levels are associated with poor overall survival of gastric carcinoma patients (30). Additional studies must be carried out to both expand the analysis to uPA-R expression and solve the discrepancy between the literature (19) and the present RT-PCR results on uPA.

In conclusion, this study shows that RT-PCR assay of small biopsy samples allows comparative evaluation (tumor and normal samples) of an entire gene complement involved in the eventual MMP-2 activity. The results demonstrate that MT1-MMP expression in gastric carcinoma is associated with poor overall survival, suggesting MT1-MMP and/or MT1-MMP: MMP-2 as new molecular-level prognostic factors.

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