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Prognostic significance of MMP-9 and TIMP-1 in liver metastases

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Abstract

Our research focuses both on the correlations between MMP-9 and TIMP-1 and classical clinicopathological factors and on the prognostic value of MMP-9 and TIMP-1 for survival. The study group included 52 patients diagnosed with hepatic metastases. The tissue specimens have been specifically processed for immunohistochemical exam, by using anti-MMP-9 and anti-TIMP-1 antibodies. For the semi-quantitative assessment, we have used an individualized score, which values allowed the discrimination of two classes of cases (low and high), using two different thresholds: ≤4 and <4. Data have been statistically analyzed by using Fisher 2×2 test and Kaplan–Meier curves. Statistical analysis between MMP-9 and TIMP-1 expression (low *versus* high, separately for each threshold) and clinicopathological characteristics had not revealed significant differences. In both types of threshold applied in survival analysis, significant differences between MMP-9 and TIMP-1 low and high expression have been demonstrated. For cases with concordant MMP-9–TIMP1 co-expression, low *versus* high, the survival analysis revealed that threshold value <4 offers a better stratification of cases when compared to threshold value ≤4, based on significant differences registered only for threshold value <4. No significant differences were registered between cases with discordant MMP-9–TIMP-1 co-expression, for both thresholds. Regardless of the used threshold, the survival analysis achieved between the cases with MMP-9–TIMP-1 concordant co-expression and cases with MMP-9–TIMP-1 discordant co-expression had proven significant differences. Our study suggests that the confirmation of MMP-9 and TIMP-1 value as prognostic factors, based on immunohistochemical expression, requires a threshold validation.

***Keywords*:** liver metastasis, MMP-9, TIMP-1, prognostic factor, threshold.

Introduction

The cellular populations, which are part of the hepatic parenchyma, being characterized by an important phenotypic heterogeneity, are responsible for the complex liver functions, reflected in their involvement in biosynthesis, metabolism, clearance and defense activities [1].

During different stages of primary and secondary hepatic carcinogenesis, these cellular components, organized in a unique microenvironment, interact with the tumor cells [1] – an important role in this dialogue is played by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).

MMPs characterization, as members of a family of Zinc-dependent endopeptidases, debuted in '90s [2]. More than 20 members of MMP genes family have been identified, categorized by structural and substrate specificity in seven main types: collagenases, gelatinases, stromelysins, stromelysin-like, matrylisins, membranar type MMPs, and other MMPs [2].

Numerous articles have been aimed to study MMPs involvement in tumor progression and metastasis, their published results supporting the recognition of MMPs role in tumor invasion and dissemination, by their capacity of degradation or even of destruction of extracellular matrix [3]. Moreover, MMPs possess the capacity to amplify the growth factors ability, without any response to their inhibition, to avoid apoptosis, stimulating the tumor cells proliferation, and to promote angiogenesis [2, 4]. Supplementary to these mechanisms, MMPs are responsible of pre-metastatic niche formation [5, 6], interrelate with the tumor associated immune infiltrate and, consequently, play a role in cancer immunity [6, 7].

MMPs activity is regulated by TIMPs, endogenous molecules, which, on their part, have regulatory action over extracellular matrix turnover, tissue remodeling, and cellular behavior [8]. It is worthwhile mentioning that, independent of MMPs inhibition, TIMPs itself may enhance cellular proliferation, may exert anti-angiogenic effects, and may have dual pro- and anti-apoptotic roles [8].

The mainstream publishers contain numerous reports of MMP-1, MMP-13, MMP-2, MMP-9, MMP-3, MMP-10, MMP-11, and MMP-7 amplified expression in various types of primary tumors [9]. Commonly, MMPs expression evaluation is achieved in correlation with TIMPs expression evaluation. However, relatively few studies are oriented toward MMPs and TIMPs analysis in liver metastases, the main focus being the colorectal hepatic metastases [10–16]. Moreover, MMPs and TIMPs prognosis factor value is poorly analyzed and current data are predominantly linked to tumor pro-angiogenic phenotype [17–25].

These premises justify our interest in MMPs and TIMPs expression characterization in liver metastatic tumors [26].

Our study develops the previous experience in interpretation of the large variability of MMPs and TIMPs in hepatic metastatic site, the current research focusing on the correlations between MMP-9 and TIMP-1 and classical clinicopathological factors, and on the prognostic value of MMP-9 and TIMP-1 for survival.

Materials and Methods

Patients

The study group included 52 patients diagnosed with hepatic metastases and surgically treated in the Surgical Clinics of "St. Spiridon" University Hospital, Iassy, Romania, without chemotherapy or radiotherapy before the surgical treatment.

The clinicopathological features of the patients are summarized in Table 1.

Table 1 – ***Clinicopathological characteristics of patients***

Clinicopathological characteristics		Cases	
		No.	*Percent*
Age [years]			
≤67		24	46.15%
>67		28	53.85%
Gender			
Female		25	48.07%
Male		27	51.93%
Tumor stage			
Stage II	T2NxM1	3	5.76%
Stage III	T3NxM1	37	71.25%
Stage IV	T4NxM1	12	23.07%
Histological grade			
G1	Well differentiated	3	5.76%
G2	Moderately differentiated	27	51.92%
G3	Poorly differentiated	14	32.69%
G4	Undifferentiated	5	9.61%
Tumoral extension			
One lobe		30	57.69%
Many lobes		22	42.31%

The primary tumor has been localized in digestive territory, as following: colorectal cancer – 33 cases, gastric cancer – 10 cases, duodenal cancer – two cases, gallbladder cancer – two cases, and pancreatic cancer – five cases. The survival information has been obtained by follow-up, all deaths being related to the cancer disease.

Tissue samples. Immunohistochemistry

The material has been represented by tissue specimens obtained from corresponding paraffin-embedded tissue blocks from the archive of the Department of Pathology, "St. Spiridon" University Hospital, Iassy.

The tissue specimens have been specifically processed for immunohistochemical exam, by using anti-MMP-9 (clone 2C3, Santa Cruz, USA) and anti-TIMP-1 (clone 102D1, Santa Cruz, USA) antibodies.

The immunohistochemical technique followed the standard working protocol: sections dewaxing and re-hydration; antigen retrieval, by heat-induced epitope retrieval technique (using Antigen Retrieval Solution pH 6 and a water bath at 98^0C for 30 minutes); endogenous peroxidase blocking (using hydrogen peroxide 3% for 10 minutes); primary antibody incubation (overnight, at 4^0C, MMP-9 1/100 dilution, TIMP-1 1/100 dilution); amplification of the immunoreaction with secondary and tertiary antibody (using LSAB-HRP complex, Dako, Carpinteria, USA); reaction developing (with 3.3'-diamino-benzidine tetrahydrochloride chromogen, DakoCytomation, Carpinteria, USA); counterstaining with modified Lillie's Hematoxylin.

Semi-quantitative assessment

According to our previous experience in MMPs and TIMPs evaluation [26], we have used an individualized score, generated by association of criteria that reflect the reaction intensity (I) and criteria that indicate the percent of positive cells (P), used in similar studies [9, 18–20, 27, 28] (Table 2).

Table 2 – ***Score' criteria***

Intensity of immunoreaction		Percent of positive tumoral cells	
Assessment	*Score*	*Percent*	*Score*
Low (+)	1	Absent	0
		<10%	1
Moderate (++)	2	10–50%	2
Strong (+++)	3	>50%	3

The final score has been obtained according to the following formula: I×P.

The obtained score values allowed the discrimination of two classes, using two different thresholds (≤4 and <4), as follows:

- cases with scores ≤4 were considered cases with low score, and cases with scores >4 – cases with high scores;
- cases with scores <4 were considered cases with low score, and cases with scores ≥4 – cases with high scores.

Statistical analysis

Statistical analysis was completed using MedCalc software (MedCalc Software, Ostend, Belgium). Fisher 2×2 test has been used for evaluation of correlation between the clinicopathological characteristics and MMP-9 and TIMP-1 expression. Survival analysis was based on Kaplan–Meier curves and *log*-rank test. $P<0.05$ was considered as statistically significant.

Results

The qualitative analysis of tumor areas immuno-reactivity for MMP-9 and TIMP-1 reflected a broad spectrum of presentations, as a mirror of an equal or unequal secretory capacity (Figures 1–4). Thus, the staining pattern has been characterized as complex, homogenous or heterogeneous.

The individual semi-quantitative analysis of MMP-9 and TIMP-1 expressions allowed the categorization of each case of the studied group into one of the two classes (low or high) established by score system application, for threshold ≤4 and <4, respectively (Table 3).

For both thresholds (≤4 and <4), the results obtained for each case demonstrated either a concordant pattern of MMP-9–TIMP-1 co-expression (both score values being high or low), either a discordant pattern (MMP-9 evaluated as low score and TIMP-1 evaluated as high score, or opposite) (Table 3).

Statistical analysis between MMP-9 and TIMP-1 expression, respectively (low score *versus* high score, separately for threshold ≤4 and <4) and classic clinico-pathological characteristics (age, tumor stage, histological grade, and tumor extension) have not revealed significant differences.

For all 52 cases, the survival analysis between cases with MMP-9 low score and MMP-9 high score established

by threshold ≤4 and <4, respectively, revealed significant differences in both circumstances (p=0.0002 and p=0.0001, respectively) (Figure 5, A and B). Similar results were obtained for TIMP-1 (p=0.001 and p=0.0025, respectively) (Figure 6, A and B).

For cases with concordant MMP-9–TIMP-1 coexpression, based on threshold ≤4, the survival analysis revealed no significant differences between low score and high score cases (p<0.34) (Figure 7A). On the other hand, when we used the threshold <4, we obtained significant differences between low score cases and high ones (p<0.0001) (Figure 7B).

The survival analysis indicated no significant differences between cases with MMP-9 low score–TIMP-1 high score and cases with MMP-9 high score–TIMP-1 low score, for both thresholds (p=0.89 and p=0.09, respectively) (Figure 8, A and B).

Regardless of the used threshold, the survival analysis achieved between the cases with MMP-9–TIMP-1 concordant co-expression (evaluated either with low, either with high score) and cases with MMP-9–TIMP-1 discordant co-expression (either MMP-9 low–TIMP-1 high, either MMP-9 high–TIMP-1 low) exhibited significant differences (p=0.007, p=0.011) (Figure 9, A and B).

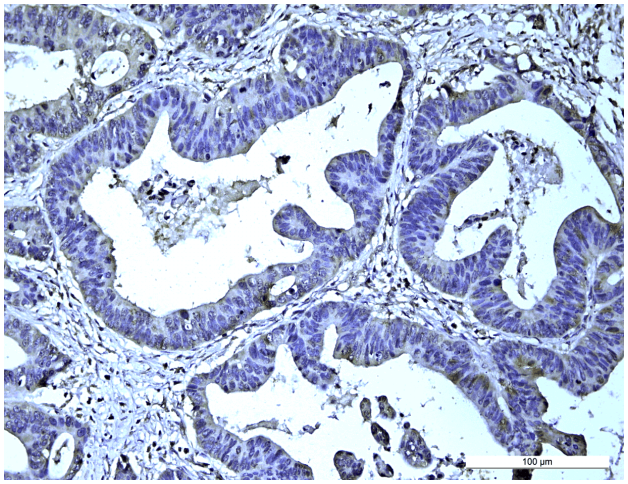

Figure 1 – ***MMP-9 expression, final score 2 (moderate staining intensity, less than 10% positive tumoral cells). IHC, 200×.***

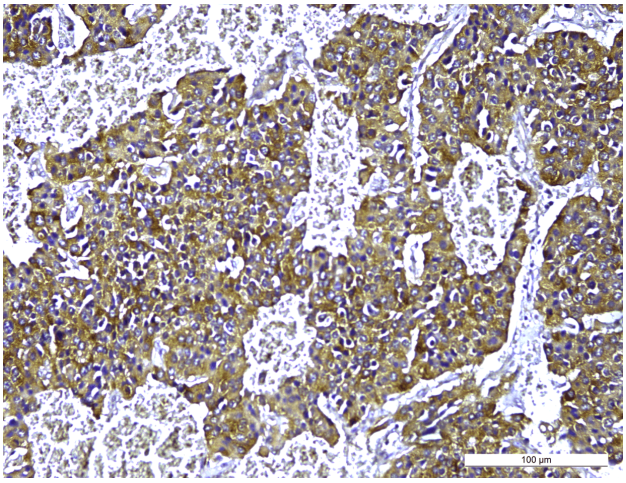

Figure 2 – ***MMP-9 expression, final score 9 (strong staining intensity, 100% positive tumoral cells). IHC, 200×.***

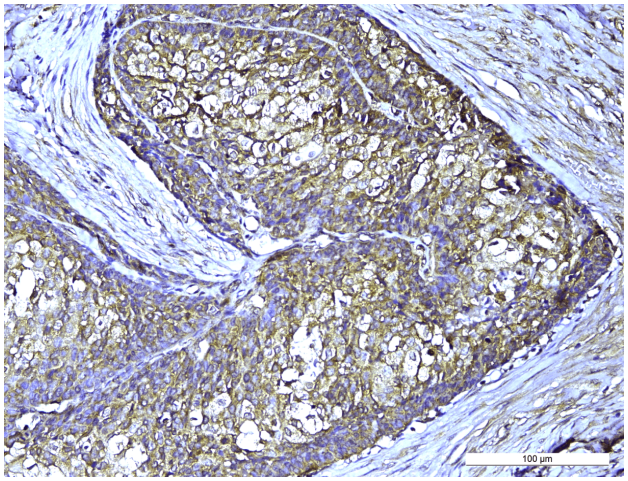

Figure 3 – ***TIMP-1 expression, final score 6 (moderate staining intensity, 100% positive tumoral cells). IHC, 200×.***

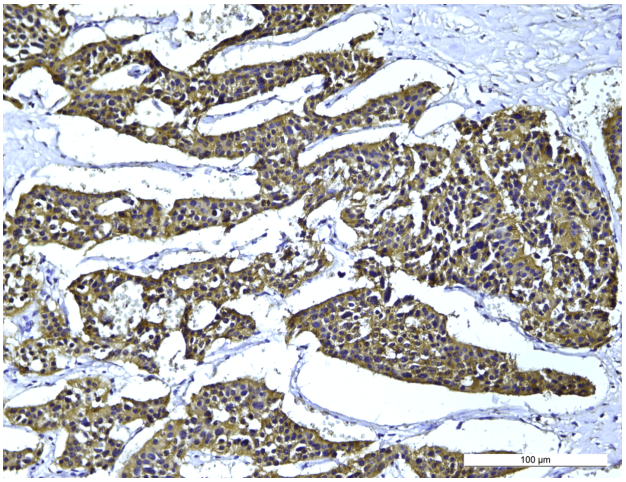

Figure 4 – ***TIMP-1 expression, final score 9 (strong staining intensity, 100% positive tumoral cells). IHC, 200×.***

Table 3 – ***Synopsis of the semi-quantitative analysis of MMP-9 and TIMP-1, by using threshold ≤4 and <4***

Threshold ≤4						Threshold <4					
MMP-9–TIMP-1 low 24 cases			***MMP-9 low–TIMP-1 high 6 cases***			***MMP-9–TIMP-1 low 13 cases***			***MMP-9 low–TIMP-1 high 5 cases***		
MMP-9 score	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*
0	0	5	3	6	1	0	0	5	0	4	2
0	2	1									
0	4	2				0	2	1			
2	0	2									

Threshold ≤4						Threshold <4					
MMP-9–TIMP-1 low 24 cases			***MMP-9 low–TIMP-1 high 6 cases***			***MMP-9–TIMP-1 low 13 cases***			***MMP-9 low–TIMP-1 high 5 cases***		
MMP-9 score	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*
2	2	1	4	6	3	2	0	2	3	4	2
2	3	1									
3	0	1				2	2	1			
3	2	2									
3	4	2				2	3	1			
4	0	2	4	9	2				3	6	1
4	2	1				3	0	1			
4	3	1									
4	4	3				3	2	2			
MMP-9–TIMP-1 high 10 cases			***MMP-9 high–TIMP 1 low 12 cases***			***MMP-9–TIMP-1 high 25 cases***			***MMP-9 high–TIMP 1 low 9 cases***		
MMP-9 score	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*	*MMP-9 score*	*TIMP-1 score*	*No. of cases*
6	6	4	6	2	3	4	4	3	4	0	2
						4	6	3			
			6	3	1	4	9	2	4	2	1
						6	4	5	4	3	1
9	6	5	6	4	5	6	6	4	6	2	3
						9	4	2			
			9	2	1	9	6	5	6	3	1
9	9	1	9	4	2	9	9	1	9	2	1

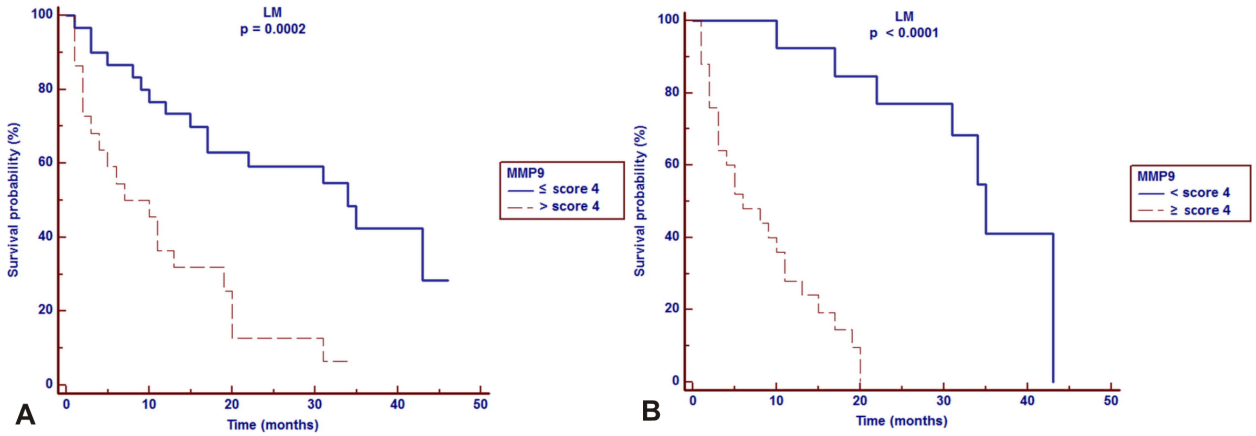

Figure 5 – *Overall survival according to MMP-9 expression: (A) Threshold ≤4; (B) Threshold <4.*

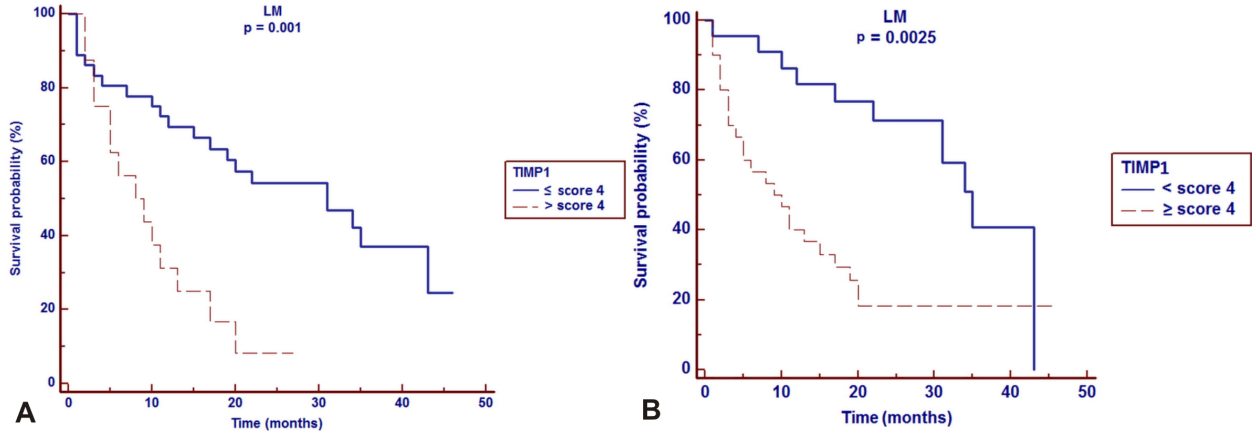

Figure 6 – *Overall survival according to TIMP-1 expression: (A) Threshold ≤4; (B) Threshold <4.*

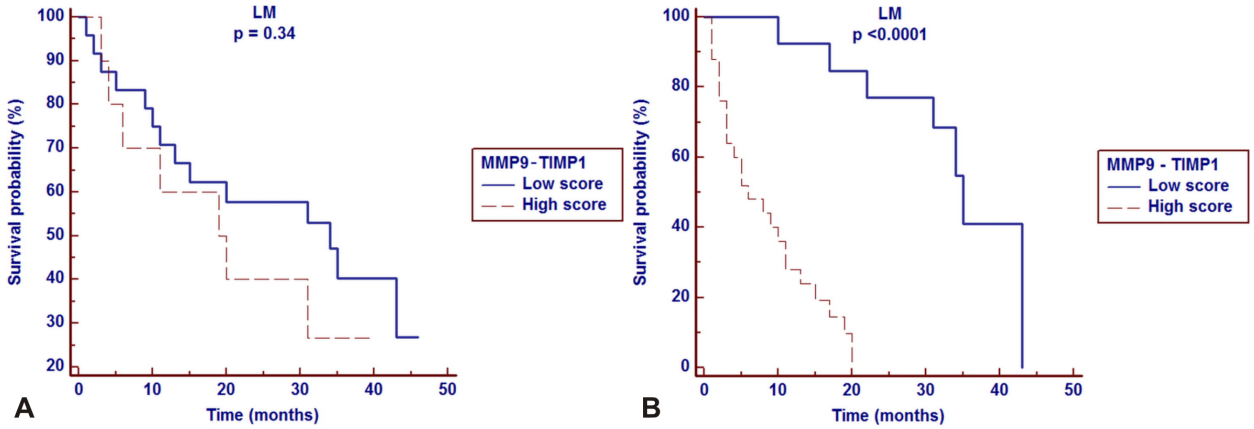

Figure 7 – ***Overall survival according to concordant MMP-9–TIMP-1 co-expression: (A) Threshold ≤4; (B) Threshold <4.***

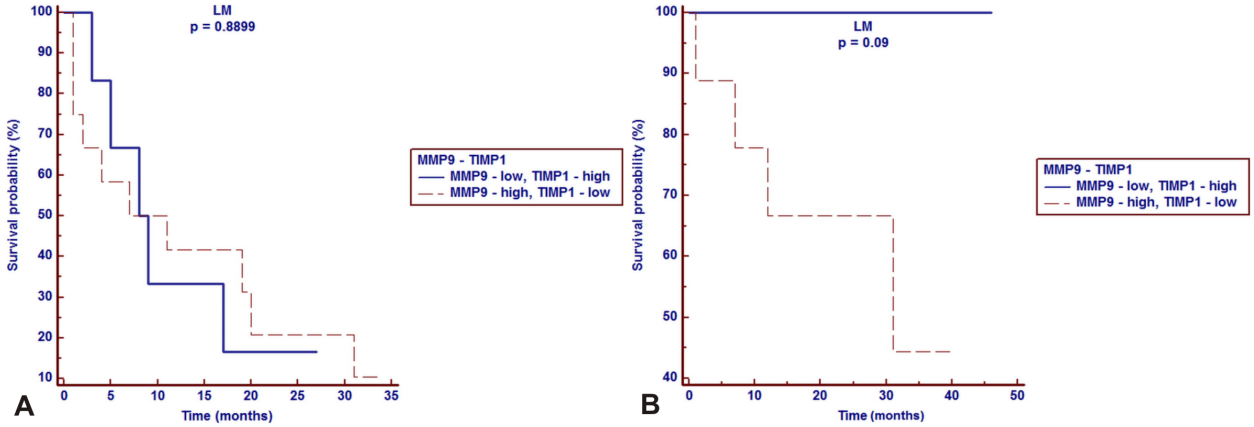

Figure 8 – ***Overall survival according to discordant MMP-9–TIMP-1 co-expression: (A) Threshold ≤4; (B) Threshold <4.***

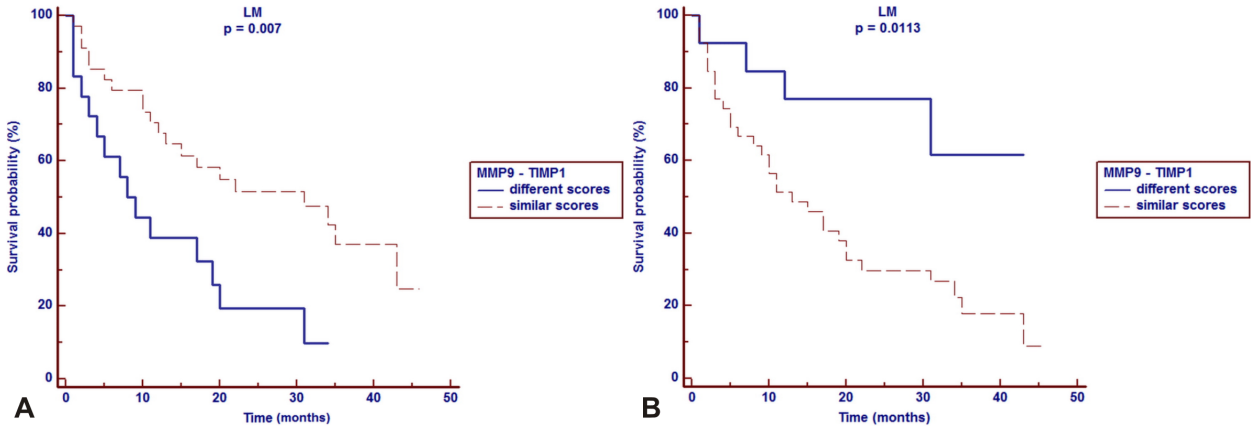

Figure 9 – ***Overall survival according to concordant versus discordant MMP-9–TIMP-1 co-expressions: (A) Threshold ≤4; (B) Threshold <4.***

Discussion

The status of MMPs–TIMPs balance represents an interesting topic in relationship with its potential prognostic value. The studies on the relationship between MMPs–TIMPs expression and survival, which have been published beginning with '90s, have been mainly focused on gastrointestinal location. The results demonstrate that increased tissue expression and serum levels of MMPs and TIMPs determine the decrease of disease-free and overall survival [29–31], as MMPs intervene in adenoma–carcinoma progression [32]. Consequently, MMPs and TIMPs seem to have an important contribution to the development of an aggressive phenotype, with negative impact on survival [18, 20].

After 2000, the mainstream publishers also include studies oriented toward MMPs and TIMPs expression in liver metastases, in an attempt to clarify if MMPs and TIMPs may be considered as useful prognosis markers for these conditions [10–16, 33, 34]. The most studies are focused on MMP-2, MMP-7, MMP-9 activity, in correlation to that of TIMP-1 and TIMP-2.

It is also worthwhile to highlight the interest in MMPs and TIMPs reactivity because of different therapeutic methods used in liver metastases, such as hepatic radio-

frequency ablation [35]. Although authors have reported an important increase in MMP-2 and MMP-9 expression in tissue adjacent to the ablation area, no significant differences have been obtained in survival, in comparison to cases treated by classical surgical resection [35].

Tissue immunohistochemical evaluation is considered as superior over the serum evaluation, in which the results may be altered by the liberation of neutrophils enzymes into the blood clot [9]. However, only few reports are based on the immunohistochemical expression of MMPs and TIMPs, and the variability in the studies' design and quantitative assessment may be the reason of results inconsistency [9, 18–20, 27, 28].

Thus, there are still gaps in understanding the action context of MMPs–TIMPs and of their prognostic impact, including dilemma over the essential role of either tumor or stromal cells in the production of these molecules [9, 36].

In our study, we have been motivated by the fact that standardized criteria do not yet exist, in order to certify MMPs and TIMPs cutoff value for negative activity initiation and significant involvement in tumor biology.

Based on the preliminary results which confirmed the high variability of MMPs and TIMPs in liver metastases [26], the novelty of our study results from the application of two threshold values, ≤4 and <4. Consequently, the score value of 4 has been firstly appreciated as low and secondly as high. This double evaluation has been implemented in order to refine the categorization level between low score, with possible favorable evolution, and high score, which may have a poor prognosis.

As a general consideration over the study group, using both threshold ≤4 and threshold <4, the general pattern of the investigated markers has been extremely heterogeneous, four types of expression being identified: both MMP-9 and TIMP-1 low, both MMP-9 and TIMP-1 high, MMP-9 low and TIMP-1 high, and MMP-9 high and TIMP-1 low.

The count of cases with concordant score values for MMP-9 and TIMP-1, respectively (either low, either high) has been larger than the count of cases with non-concordant values. In each class, either low or high, MMP-9 and TIMP-1 identical score values have been occasional findings. This observation is a mirror of the opposite relationship between MMP expression and its correspondent TIMP.

MMP-9 increased expression along with TIMP-1 reduced expression are indicators of an increased aggressiveness potential, due to proteolytic capacity which facilitates the invasivity, while reduced MMPs expression and more amplified TIMPs expression primarily signify an inhibitory effect of invasive capacities. Moreover, MMPs and TIMPs expression variability depends on promoter or inhibitory action of stromal and/or tumor cells whom behavior should be integrated in the complex biochemical interferences of the tumor-associated microenvironment [9, 36].

Supplementary, updated reports certify the relationship between MMPs and angiogenesis, MMPs possessing the capacity to modulate VEGF bioavailability in tumor microenvironment [37, 38].

The particularity of our study consists in the correlation between the MMPs–TIMPs expression assessed in accordance with the two thresholds, and survival.

Thus, in our first evaluation, we have considered cases with low score ≤4 and cases with high score >4, whereas the second evaluation have been achieved by dividing the cases with low score <4 from cases with high score ≥4. Our comparative results showed that we have thus obtained a better characterization of studied cases, exhibiting a closer correlation between biological behavior and survival.

In both types of threshold applied in survival analysis, significant differences between MMP-9 low and high, and between TIMP-1 low and high expression have been demonstrated. However, the results obtained from survival analysis of cases exhibiting concordant MMP-9–TIMP-1 co-expression, low *versus* high, indicates the fact that threshold value <4 offers a better stratification of cases when compared to threshold value ≤4. This assumption is based on the statistically significant differences that have been registered for threshold value <4. Thus, we appreciate the fact that score 4, which reflects MMPs and TIMPs positivity starting with 10% of tumor cells and moderate intensity immunoreaction at least, has to be interpreted as high score, with consequent prognosis influences.

The lack of statistically significant data obtained by survival analysis performed for cases characterized by discordant MMP-9–TIMP-1 co-expression deserves a particular discussion. Based on the certified MMPs–TIMPs unbalance role in tumor progression, we would expect significant differences between cases exhibiting MMP-9 low–TIMP-1 high and cases presenting MMP-9 high–TIMP-1 low scores.

The lack of these differences signifies our insufficient knowledge of the modulation of MMPs production and action through TIMPs, this angle of research being still open.

Within this context, a highlight on the certification of dual TIMP-1 role, not only in MMPs modulatory activity, but also of MMPs independent function should be made [39].

Our double evaluation has revealed that threshold <4 is superior in comparison to threshold ≤4. At a glance, the Kaplan–Meier curve in Figure 8B) indicates a survival probability of 100% in cases exhibiting MMP-9 low–TIMP-1 high expression, as all the patients are alive, in comparison to the variability of survival probability in cases with MMP-9 high–TIMP-1 low expression, which includes both alive and dead patients. Within this context, we are aware of the necessity of study extension in order to validate the threshold value <4 in patients' stratification, with favorable and poor prognosis, respectively.

Threshold <4 application, in comparison to threshold ≤4, offers a different perspective in survival results interpretation of patients with consistent MMP-9–TIMP-1 scores (low–low or high–high) *versus* discordant scores (low–high, high–low). Practically, threshold <4 (see Figure 9B) attests the beneficial impact of counterbalance in MMP-9–TIMP-1 expression, by better overall survival and survival probability compared to cases stratification by threshold ≤4 (Figure 9A). In our opinion, this remark

suggests, based on the functional MMP-9–TIMP-1 tandem, the MMP activity limitation by the control exerted by TIMPs.

Strictly referring to the correlation between MMP-9–TIMP-1 expression and classic clinicopathological characteristics (age, tumor stage, histological grade, and extension), no statistically significant results suggest the influence of multiple factors upon the metastatic process, thus being impossible to consider MMP-9 and TIMP-1 as independent prognostic factors.

Within this context, we may reiterate the previous statement regarding the specific MMP-9 and TIMP-1 expression identified in the working group, undoubtedly influenced by the particularities of each hepatic-associated microenvironment.

Conclusions

Our study supports MMP-9 and TIMP-1 potential to influence the tumor progression in liver metastases. The confirmation of MMP-9 and TIMP-1 value as prognostic factors, based on immunohistochemical expression evaluation, requires a threshold validation.

Conflict of interests

The authors declare that they have no conflict of interests.

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