Assessment of BPV-1 Mediated Matrix Metalloproteinase Genes Deregulation in the In Vivo and In Vitro Models Designed to Explore Molecular Nature of Equine Sarcoids

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Abstract: Matrix metalloproteinases (MMPs) represent a family of enzymes capable of biocatalytically breaking down the structural and functional proteins responsible for extracellular matrix (ECM) integrity. This capability is widely used in physiological processes; however, imbalanced MMP activity can trigger the onset and progression of various pathological changes, including the neoplasmic transformation of different cell types. We sought to uncover molecular mechanisms underlying alterations in transcriptional profiles of genes coding for MMPs, which were comprehensively identified in equine adult dermal tissue bioplates, sarcoid-derived explants, and ex vivo expanded adult cutaneous fibroblast cell (ACFC) lines subjected to inducible oncogenic transformation into sarcoid-like cells. The results strongly support the hypothesis that the transcriptional activity of MMP genes correlates with molecular modifications arising in equine dermal cells during their conversion into sarcoid cells. The alterations in MMP transcription signatures occurs in both sarcoid tissues and experimentally transformed equine ACFC lines expressing BPV1-E4′E1 transgene, which were characterized by gene up- and down-regulation patterns.

Keywords: matrix metalloproteinase; sarcoid; skin neoplasia; NGS; cDNA microarray

1. Introduction

Matrix metalloproteinases (MMPs) are proteins responsible for the disassembly of the extracellular matrix (ECM), which allows for cell migration and the release of various signaling factors. MMPs can regulate growth factors, whose activity is required to mediate different processes at a given tissue development stage [1,2]. Depending on their specific substrate, these proteins can be divided and assigned into six classes: collagenases, gelatinases, stromelysins, matrilisins, membrane-type MMPs, and other MMPs [1]. A wide variety of MMPs are involved in many physiological processes that are indispensable for remodeling of the tissue environment, e.g., wound healing. Importantly, the destabilization
of their expression pattern in the tissue can lead to the promotion of many pathological changes such as intestinal inflammation in humans or recurrent airway obstruction and laminitis in horses, and various tumors (i.e., lung cancer, liver metastases, and gastric carcinomas) [3]. Moreover, MMPs are believed to incur the initiation and progression of neoplastic transformation processes by breaking down the ECM proteins. Related to the degradation function of ECM is the ability of cancer cells to metastasize. The breakdown of ECM and the various growth factors, cytokines, and chemokines released as a result of this process make metalloproteinases involved in every step of metastasis, starting with the induction of epithelial to mesenchymal transition (EMT), resistance to anoikis, supporting the angiogenesis process, and activating cancer cells invasion and colonization of new tissues. Therefore, metalloproteinases are considered to be one of the major supportive factors for cancer. The large influence of metalloproteinases on metastasis, a process that is the most common cause of death from cancer, makes them an important aspect of research in the context of cancer development [4,5]. However, molecular pathways regulating the transcriptional activity of particular genes in this family have not been precisely recognized so far. Some MMPs have been found to be over-expressed in neoplastic tissues, and others have been shown to be over-expressed in healthy tissues as a result of a natural response to inflammatory processes [3].

The most common skin tumor in the Equidae family is the sarcoid (Figure 1). It manifests itself in many forms, which vary according to the extent of severity, and it can decrease the quality of animal life. It has been established that sarcoids do not metastasize [6]. There is general agreement that bovine delta-papillomaviruses induce sarcoids, i.e., bovine papillomaviruses types 1 and 2, and possibly 13 (BPV1, BPV2, and BPV13) [7,8], but the molecular nature of the BPV oncoprotein-associated tumor formation process is not entirely understood.

![Figure 1. Example of sarcoid lesion—sarcoid surgically removed from a two-year-old horse (noble half-blood horse); sarcoid was localized on gaskin (by Witkowski M).](image-url)

Therefore, research targeted to comprehensively unravel the role of MMP expression and deregulation in the oncogenic transformation of equine dermal cells into sarcoids and their spread is imperative. Consequently, the objective of the current study was to explore the transcriptional profile of a panel of MMP genes in healthy equine skin and sarcoid tumors as well as in expanded adult cutaneous fibroblast cells (ACFCs) transfected with BPV1-E4E1 fusion gene, which can be considered as an in vitro model for one of the development steps of sarcoid disease.
2. Materials and Methods

2.1. MMP Genes Selection

The experimental workflow is presented in Figure 2. The high-throughput analysis of transcriptomic signatures [9] was used to identify the MMP genes for further analyses based on applying cDNA microarrays to compare the gene expression patterns in healthy skin and sarcoid tissue samples. Additionally, using the RNA-seq technique, a panel of studies was accomplished to estimate the MMP gene expression profiles in both BPV-E4’E1 transfected ACFC lines and their counterparts transfected with empty vectors as a negative control group [10]. Considering both data sets generated (designated as GSE83430 and GSE193906), genes that belonged to the MMP family and their corresponding fold change values were achieved. The data obtained from microarrays were reanalyzed in a way similar to previous work [9], however without final filtering of genes by fold-change ($p$-value < 0.05). The RNA-seq data were obtained via sequencing of cDNA libraries on NextSeq 500 Illumina platform (Illumina) using NextSeq 500/550 High Output KIT v 2.5 (75 cycles) and according to protocol. The libraries were prepared using TruSeq RNA Kit v2 kit (Illumina) and evaluated using Qubit fluorimeter (Invitrogen, Thermo Fisher, Santa Clara, CA, USA). The obtained reads were mapped to EquCab3 reference genome (STAR software v 2.7.8). Next, the mapped reads were annotated and counted to specific gene thresholds using Ensembl gtf file version 100 (via htseq-count software v 0.13.5). Differential expression analysis was performed with the use of Deseq2 software (significance showed as $p$-value < 0.05 after multiple testing corrections).

![Figure 2](accessed on 31 March 2022) [11].

2.2. Sample Preparation

The biological samples comprised (i) equine sarcoid explants ($n = 10$) collected during standard veterinarian tumor removal, (ii) healthy skin biopptates ($n = 8$) collected at a local slaughterhouse, and (iii) ex vivo expanded ACFC lines. For all samples, the presence of BPV1 and 2 DNA was validated by PCR (AmpliTaq Gold™ 360 Master Mix; Thermo Fisher; according to the protocol) using specific primers as previously reported by Teifke et al. [12]. Skin biopptates scoring positive for BPV1 and 2 DNA were excluded from further experiments. The established ACFC lines were allotted to two groups: control cell lines transfected with an empty vector (pcDNA4/TO/myc-his/B; $n = 8$) and cell lines transfected with BPV1-E4’E1 gene constructs (pcDNA4/E4’E1/TO/myc-his/B; $n = 4$). The detailed procedures applied to establish ACFC lines and transfectant cells described by Podstawski et al. [10]. In short, fibroblast lines derived postmortem from equine skin were nucleofected with two plasmids included in the T-REx kit (Invitrogen, Waltham, MA, USA, Thermo Scientific): pcDNA™ 6/TR and pcDNA™ 4/TO/myc-His/B (with or without (negative control) ligated BPV1-E1’E6 gene construct). Nucleofection procedure
was performed with Amaxa<sup>TM</sup> Normal Human Dermal Fibroblast– Adult (NHDF-Adult) Nucleofector<sup>TM</sup> Kit (Lonza, CELLLAB, Warsaw, Poland). Positively transfected cell lines were obtained during antibiotic selection with 200 µg/mL zeocin (Invitrogen) and 6 µg/mL blasticidin S (Thermo Scientific, Waltham, MA, USA), which was held for one week.

2.3. Quantitative PCR Analysis

The conditions and parameters of qPCR method were thoroughly specified in our previous report [13]. Briefly, the RNA was isolated with PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, according to the protocol). The quality and quantity of obtained RNA were checked using TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA) and RNA Screen Tape (Agilent Technologies, Santa Clara, CA, USA). cDNA strand synthesis was performed with High-Capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Scientific, according to the protocol). The qPCR was carried out with AmpliTaq 5× HOT EvaGreen qPCR Mix Plus (ROX) (BIOTUM, Novazym, Poznań, Poland, according to the protocol) and specific primers (Table 1, Figure 3). Each sample was performed in three replications. Obtained results were calculated with the \( \Delta\Delta CT \) method [14] based on two endogenous controls: β-actin (ACTB) and ubiquitin B (UBB) [15].

Table 1. Specific primer sets used for amplification of MMPs cDNA. We investigated specific splice isoforms and exons spans.

| Gene   | Accession Number | Primers                                           | Product Length [bp] | Localisation | Splice Variants | Splice Variants Accession Number |
|--------|------------------|---------------------------------------------------|---------------------|--------------|-----------------|----------------------------------|
| MMP1 V1V2 | ENSECAG000000023733 | F: GCTGAAAGTGACTGGGAAGC R: GTCCCATCCTGCTTGTGT | 179                 | Exon 2 and 3 | 2/3             | ENSECAT00000022856.2 |
| MMP1 V3  | ENSECAG000000023733 | F: CCCCAGTGGAAAGCAGAAATAA R: ATCCATGGTCCTCATCAA | 224                 | Exon 12 and 14 | 1/3             | ENSECAT00000025715.2 |
| MMP2     | ENSECAG00000000953  | F: TCCCTTCTCCTCAACAGGC R: CGTTATTCGCCTCTGTGC | 112                 | Exon 4       | 2/2             | ENSECAT00000044147.1 |
| MMP9     | ENSECAG00000013081  | F: CGGTTCCTCCACTCCCGG R: GGTTGATGTTGCGGATGT | 141                 | Exon 6       | 3/3             | ENSECAT00000056131.1 |
| MMP12 V1V2 | ENSECAG000000019445   | F: TGGACATGTGCACAGACCCT R: CAGCCCTGCTGGTTATCC | 222                 | Exon 2 and 3 | 2/3             | ENSECAT00000022326.3 |
| MMP12 V3  | ENSECAG000000019445  | F: GATCATTCGAAGGAGTACG R: GCATCCGAGACGTCAGT | 194                 | Exon 11 and 12 | 1/3             | ENSECAT00000020742.2 |
| MMP13    | ENSECAG00000005506   | F: GCCTCCGAGAAATGCAGTCTT R: AGGGATAGCGGTCATTGG | 140                 | Exon 2       | 1/1             | ENSECAT00000070392.1 |
| MMP14    | ENSECAG000000008351  | F: CAGATCTTCTCGTCCAGG | 109                 | Exon 4       | 1/1             | ENSECAT0000008899.3 |
| MMP17    | ENSECAG000000013201  | F: TATCATGGCCCTCAGGTTTC | 118                 | Exon 4       | 2/2             | ENSECAT00000013924.2 |
| MMP27    | ENSECAG000000019404  | F: CCGTGACTGGAAAACACTGT | 238                 | Exon 2 and 3 | 1/1             | ENSECAT00000020635.3 |

Figure 3. Schematic presentation of primers designed for MMP1 and MMP12 genes to amplify all splicing variants. MMP–201–203 are splicing variants according to Ensembl accession number (MMP1-ENSECAG000000023733; MMP12-ENSECAG00000019445); primer amplification region is presented as an orange rectangle, and blue boxes represent exons.
2.4. Statistical Analysis

The obtained RQ values were firstly tested for distribution purposes with a Shapiro–Francia normality test followed by Mann–Whitney U-test to assess the inter-group variability. Both tests were carried out using R software v 4.1 [16].

3. Results and Discussion

The present study was designed to determine changes in the MMP gene expression profiles that can be positively correlated with the development of equine sarcoids. For this purpose, to comprehensively evaluate the molecular patterns of sarcoïd-dependent neoplastic transformation, the in vivo and in vitro models have been applied. To comparatively examine the transcriptomic signatures of MMP genes, the expression profile in equine sarcoïd tissue samples and BPV1-E4’E1 nucleofected ACFC-derived tumor cells were compared to healthy dermal tissues and ACFC lines not subjected to oncogenic transformation. First, the high throughput data was used to detect the MMP genes expressed in the investigated groups of cells and tissues and to demonstrate significant differences between them. The comparative analysis of MMP genes between sarcoïd and healthy skin tissue samples allowed us to detect a total of 21 genes belonging to the MMP family, out of which three representatives (MMP2, MMP7, and MMP23B) underwent significantly differential expression patterns (Figure 4A). In turn, the whole transcriptome sequencing-mediated analysis of BPV1-E4’E1 transgenic equine ACFC-derived neoplastic cell lines and their counterparts nucleofected with empty vectors revealed the expression of 16 MMP family genes, out of which six members (MMP1, −9, −12, −17, −19, and −27) showed significantly differential expression (Figure 4B).

Next, the expression profiling using qPCR confirmed modifying the transcriptional activities for MMP genes representing both in vitro and in vivo models of sarcoïd-dependent oncogenic transformation. Additionally, for two genes (MMP1 and MMP12), the splice vari-
ant analysis was also performed to prove the possible involvement of transcripts encoding different MMP isoforms in the processes of either transgenically induced neoplastic transformation in BPV-E4'E1 nucleofected ACFCs into sarcoid-like cells or pathophysiological conversion of dermal tissues into sarcoïds.

Expression of several MMP genes was already investigated in equine sarcoïds in terms of ECM remodeling [18]. It was hypothesized that ECM remodeling is a pivotal step of etiopathogenesis of sarcoïd-dependent neoplasia of equine dermal tissues that provides clear evidence for the occurrence of aberrant expression of MMP genes. Martano et al. have also proven that the altered transcriptional activities of MMP and TIMP genes can lead to imbalance between collagen synthesis and degradation processes and, consequently, can result in skin neoplasia progression [18]. In turn, the study results by Yuan et al. [16] have confirmed that MMPI, −2, and −9 genes were up-regulated in sarcoïd tumors and their over-expression was highly associated with the invasion fibroblast-derived sarcoïd cells. Compared to the published data, we provide strong evidence of significant differences in the expression levels of MMP2 and MMP9 genes, which turned out to be up-regulated in both the ex vivo and in vitro model of sarcoïd-specific neoplastic transformation (Figure 5). Furthermore, an augmented transcriptional activity of MMPI7 gene was also observed in dermal sarcoïd samples and BPV1-E4'E1 nucleofected ACFC derivatives. MMPI7 belongs to genes, whose enhanced expression has been found to be related to onset and progression of inflammatory processes. Therefore, it’s up-regulation may be a biomarker of inflammation evoked in sarcoïd tissues and oncogenically transformed cells [19].

![Figure 5](image-url)

Figure 5. The differences in MMP gene expression levels between analyzed groups of equine sarcoïds (ES), skin (SK) samples, control ACFC lines nucleofected with empty vector (FL), and ACFC lines nucleofected with BPV-E4'E1 transgene (BPV) (R software v 4.1) [16].

The whole transcriptome analysis on the in vitro model reflecting BPV1 infection in equine dermal fibroblast cells confirms the remarkable deregulation of MMP genes in BPV1-E4 and BPV1-E4'E1 transgenic ACFC neoplastic cell lines [10]. In 2008, Yuan et al. [20] evaluated the impact of BPV1 on the transcriptional profile of equine fibroblast cell lines. They pinpointed that the MMPI gene was one of the most deregulated gene associated with the virus infection. Taking into account different splice isoforms of the MMPI gene, the present research has identified a significant down-regulation of this gene in sarcoïd tissue samples and nucleofected ACFC lines. Such modification was the most evident for V3 variant designated as ENSECAT00000025715.2 (fold changes equal to −5.90 and −12.26; p-values < 0.05 and < 0.01, respectively) (Figure 5). Moreover, as depicted in Figure 5, a similar expression profile was detected for all analyzed MMPI2 isoforms, which were characterized by significantly and insignificantly diminished levels of V3 variant termed as ENSECAT00000020742.2 in sarcoïd tissue explants (fold change equal to
−1.95; *p*-value < 0.05) and BPV1-E4′E1 nucleofected ACFC lines (FC 1.31; *p*-value ≥ 0.05), respectively. This can indicate that BPV1 infection contributes to diminished MMP1 and MMP12 expression levels. Moreover, different involvement of individual splice variants may suggest the importance of their individual roles in the neoplastic process.

Interestingly, transcription of three other genes—MMP13 (*p*-value < 0.05), MMP14 (*p*-value < 0.001), and MMP27 (*p*-value ≥ 0.05)—was down-regulated in sarcoïd tissue samples as compared to healthy skin bioptries. Observed MMP27 down-regulation contrasts with the results of other studies performed on tumors, where MMP27 mRNA was equally expressed in normal breast and breast cancer tissues [21]. These findings can support the hypothesis that MMP27 protein can play an important role in oncogenic transformation in a variety of tumor types. In turn, down-regulation of MMP13 and MMP14 genes can be one of the most important reasons for sarcoïds’ very or negligibly low capability to metastasize, since MMP14 gene silencing brings about the negative control of cell migration [22]. In contrast, the MMP13 gene is considered to be a poor prognostic biomarker related to cell migration in several cancer types (i.e., colorectal cancer and breast cancer) [23,24].

4. Conclusions

To conclude, the present report strongly supports the hypothesis that the transcriptional activity of MMP genes is correlated with molecular modifications arising in equine dermal cells during their molecular conversion into sarcoïd cells. The alterations in the MMP transcriptomic signatures that were shown to occur in sarcoïd tissues and transformed equine ACFC lines expressing BPV1-E4′E1 transgene were characterized by both MMPs up- and down-regulation patterns. Moreover, for MMP1 and MMP12 genes, significant involvement of selected splice variants may suggest that only selected isoforms are crucial for the growth and development of sarcoïds.

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