Acute phase proteins are synthesized in response to injury, such as that associated with infection, trauma, ischemia, or neoplasia. Acute phase proteins have been detected in synovial fluid from people and horses with inflammatory joint disease, but their role in such disease processes has yet to be elucidated.

Serum amyloid A is classified as a major acute phase protein in all species investigated so far (with the exception of rats) because its serum concentrations increase several hundred- or even thousand-fold in response to aseptic or septic inflammation. Synthesis of SAA occurs mainly in the liver, where production is induced by proinflammatory cytokines released at the site of inflammation. However, studies have shown that extrahepatic tissues can also synthesize SAA in response to inflammation and that isoforms of the protein expressed locally in inflamed tissues differ from those synthesized in the liver. In most species, including horses, the locally produced SAA homolog is the SAA3 isoform. Studies of humans and rabbits have shown that synoviocytes and articular chondrocytes synthesize SAA, and in 1 study, a cDNA array of equine chondrocytes revealed a 4-fold increase in SAA mRNA after 6 hours of IL-1β exposure. Intraarticular SAA has been implicated in increased activity of MMPs, which cause degradation of intraarticular structures, and studies in people have indicated that SAA protein has proinflammatory properties. Therefore, SAA protein might be linked to develop-
development of pathological changes in inflammatory joint disease.

The objective of the study reported here was to elucidate potential functions of SAA protein in equine joint disease. Our approach was to investigate production of SAA mRNA and protein in cultured equine articular chondrocytes and FLSs in response to stimulation with recombinant proinflammatory cytokines and to evaluate the effects of recombinant SAA on expression of MMPs and other inflammatory markers in both cell types.

Materials and Methods

Sample
All samples used in the study were collected with owner consent from 4 horses for each experiment (2.5 to 13 years of age) that were euthanized for reasons (not related to the study) other than lameness or orthopedic disease. Horses were euthanized by captive bolt followed by exsanguination according to regulatory guidelines. Tissues were collected aseptically from both metacarpophalangeal joints immediately after euthanasia; samples were pooled for each horse. Further processing was initiated ≤ 1 hour after sample collection.

Articular cartilage was harvested from the articular surface of the distal third metacarpal bone. These samples were obtained from 4 horses (2.5 to 6 years of age) with no signs of joint disease evident on macroscopic inspection. Chondrocytes were isolated by sequential digestion of fresh cartilage at 37°C essentially as described by Kuettner et al19; tissues were covered with high-glucose (4.5 g/L) DMEM containing penicillin G (sodium salt) (300 U/mL), streptomycin sulfate (100 µg/mL), and gentamicin sulfate (50 µg/mL) with addition of 0.1% pronase for 1 hour, followed by treatment with 0.15% collagenase Type II solution for 18 hours. Chondrocytes were cultured in monolayer in 6-well culture dishes at a density of 10⁶ cells/well in a controlled humidified atmosphere (37°C with 5% CO₂ in high-glucose DMEM with 10% fetal calf serum, ascorbic acid (50 µg/mL), penicillin (300 U/mL), streptomycin (100 µg/mL), nystatin (100 U/mL), and gentamicin (50 µg/mL) added. Culture medium was changed 2 days after the cultures had been established, and on day 3 culture conditions were changed to serum free by replacing the medium with the described solution without addition of fetal calf serum (24 hours prior to cytokine treatment).

Figure 1—Results of gene expression analysis for SAA in equine chondrocytes cultured with recombinant human IL-1β (50 ng/mL; A), recombinant equine TNF-α (50 ng/mL; B), recombinant equine IL-6 (50 ng/mL; C), or IIT (20 ng of each cytokine/mL; D) for predetermined time points up to 48 hours. Expression of SAA mRNA increased significantly over time in response to stimulation with IL-1β (P = 0.001), TNF-α (P = 0.050), IL-6 (P = 0.021), or IIT (P = 0.005). The GAPDH-normalized mRNA expression (measured by quantitative real-time PCR assay) is expressed as a percentage of that in unstimulated control cells of the same type harvested at each time point. Notice that the y-axis scale varies among figure subparts. Data are expressed as mean; error bars represent SD.

Figure 2—Results of gene expression analysis for CD-RAP in equine chondrocytes cultured with recombinant human IL-1β (50 ng/mL; A), recombinant equine TNF-α (50 ng/mL; B), recombinant equine IL-6 (50 ng/mL; C), or IIT (20 ng of each cytokine/mL; D). Expression of CD-RAP mRNA decreased significantly over time in response to IL-1β (P = 0.039) or IIT (P = 0.050) treatment; changes were nonsignificant for other treatments. See Figure 1 for remainder of key.
Similarly, synovial membrane was dissected from the lateral and medial aspects of the metacarpophalangeal joint capsules. These samples were obtained from 4 horses (10 to 13 years of age) that had no signs of joint disease detected by macroscopic inspection. Tissue samples were digested for 3 hours in high-glucose DMEM containing penicillin (300 U/mL), streptomycin (100 μg/mL), and gentamicin (50 μg/mL), with addition of 0.1% collagenase Type I. Synoviocytes were expanded in monolayer culture in the same culture medium described for chondrocytes, except that ascorbic acid was not added. At passage 3, the cells were transferred to 6-well culture dishes at a density of 10^6 cells/well. After 24 hours, the medium was changed to as described to create serum-free conditions.

**Cytokine treatment of chondrocytes and FLSs**

Cytokine treatment was initiated 24 hours after establishment of culture in serum-free medium. Chondrocytes were cultured in conditioned medium for 0, 3, 6, 12, 24, or 48 hours. Fibroblast-like synoviocytes were cultured in conditioned medium for 0, 12, 24, or 48 hours. The 0-hour samples were collected immediately before the addition of conditioned medium. The conditioned medium contained either recombinant human IL-1β (50 ng/mL), recombinant equine TNF-α (50 ng/mL), recombinant equine IL-6α (50 ng/mL), or a combination of these 3 cytokines, each at a concentration of 20 ng/mL (termed IIT). Unstimulated control cultures were kept in culture medium without cytokines. All cultures for all time points were performed in duplicate. At each time point, culture medium (1 mL/well) was collected, centrifuged at 10,000 × g for 10 minutes, and stored at −80°C until used for RNA isolation for evaluation of gene expression.

**Serum amyloid A protein detection**

Concentrations of SAA protein in lysed chondrocytes and FLSs and in cell culture medium samples obtained with a commercially available kit according to the manufacturer’s instructions. First-strand cDNA was synthesized from 0.5 μg of total RNA. Species-specific intron spanning primers were used (Appendix), and PCR amplification products were verified by sequencing with a previously described efficiency corrected calculation method as follows:

\[
NRR = \frac{E_{\text{CT (target calibrator)}} - CT (\text{target sample})}{E_{\text{CT (reference calibrator)}} - CT (\text{reference sample})}
\]

where NRR represents the normalized relative ratio, E is the efficiency coefficient for the target gene, E_r is the efficiency coefficient for the reference gene, and CT is the PCR cycle threshold value. Gene expression results were normalized to GAPDH.

**Gene expression analyses**

Total cellular RNA was extracted from harvested cells with a commercially available kit according to the manufacturer’s instructions. First-strand cDNA was synthesized from 0.5 μg of total RNA. Species-specific intron spanning primers were used (Appendix), and PCR amplification products were verified by sequencing with a commercially available kit according to the manufacturer’s instructions with a genetic analyzer. A quantitative real-time PCR assay was performed with a master mix containing cyanine dye for detection of nucleic acid and a real-time PCR system. Results were calculated with a previously described efficiency corrected calculation method as follows:

\[
NRR = \frac{E_{\text{CT (target calibrator)}} - CT (\text{target sample})}{E_{\text{CT (reference calibrator)}} - CT (\text{reference sample})}
\]

where NRR represents the normalized relative ratio, E is the efficiency coefficient for the target gene, E_r is the efficiency coefficient for the reference gene, and CT is the PCR cycle threshold value. Gene expression results were normalized to GAPDH.

**Serum amyloid A treatment of chondrocytes and FLSs**

Treatment with recombinant human SAA (1 μg/mL) was initiated after 24 hours of culture in serum-free medium. Cells of each type were cultured in the conditioned medium for 0, 4, 12, 24, or 48 hours. Cultures of cell type treated with recombinant human IL-1β (50 ng/mL) were included as respective positive controls in this part of the experiment. Unstimulated (negative) control cultures of the same cell types were maintained in serum-free culture medium without SAA or IL-1β. All cultures at all time points were performed in duplicate. At each time point, cells were harvested with the 0.05% trypsin-EDTA protocol as described for cytokine-treated cultures and subsequently used for RNA isolation for evaluation of gene expression of SAA, MMP-1, MMP-3, and IL-6 and for detection of SAA protein. In chondrocytes, CD-RAP gene expression was also assessed.

**Gene expression analyses**

Total cellular RNA was extracted from harvested cells with a commercially available kit according to the manufacturer’s instructions. First-strand cDNA was synthesized from 0.5 μg of total RNA. Species-specific intron spanning primers were used (Appendix), and PCR amplification products were verified by sequencing with a commercially available kit according to the manufacturer’s instructions with a genetic analyzer. A quantitative real-time PCR assay was performed with a master mix containing cyanine dye for detection of nucleic acid and a real-time PCR system. Results were calculated with a previously described efficiency corrected calculation method as follows:

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**Serum amyloid A protein detection**

Concentrations of SAA protein in lysed chondrocytes and FLSs and in cell culture medium samples ob-
tained before (0 hours) and 12, 24, and 48 hours after addition of cytokine-supplemented media were determined by a previously described turbidimetric immunnoassay. Detection limit of the assay was 0.48 mg/L.

To identify SAA protein isoforms, denaturing isoelectric focusing and western blotting procedures were carried out in a representative subset of the samples as described previously. The subset included pooled lysed chondrocytes from 4 horses treated for 48 hours with cytokines in vitro, pooled culture supernatant from chondrocytes of 4 horses treated for 48 hours with cytokines in vitro, and pooled chondrocyte culture supernatant from untreated control cultures of chondrocytes from 4 horses. Briefly, samples were diluted in 8M urea and separated by isoelectric focusing on dried gels rehydrated with a mixture of 8M urea and preblended isoelectric focusing media (pH 3.5–9.5) according to the manufacturer’s instructions. After separation, semidy western blotting onto a nitrocellulose membrane was performed, and SAA was stained using a biotinylated, monoclonal anti-human SAA antibody cross-reacting with equine SAA. Serum and synovial fluid samples with known high concentrations of SAA were included as positive controls on all gels.

Statistical analyses

Gene expression normalized to GAPDH was used for the statistical analyses. Overall changes in gene expression values over time (ie, effect of treatments over time) were analyzed with the nonparametric, repeated-measurements Friedman test. Values of $P \leq 0.05$ were considered significant.

To allow comparison of gene expression values between individual experiments, GAPDH-normalized gene expression in treated samples relative to that of the unstimulated control sample was determined at each time point. The mean of multiple replicates were used in analyses, and data were reported as mean ± SD.

Results

Effects of cytokine stimulation on chondrocytes and FLSs

In equine chondrocytes, expression of SAA or CD-RAP mRNA did not change significantly over time in unstimulated control cultures. In FLSs, changes in expression of SAA, MMP-1, and MMP-3 mRNA in unstimulated control cultures over time were also not significant. In chondrocytes, expression of SAA mRNA increased significantly over time in response to stimulation with IL-1β ($P = 0.001$), TNF-α ($P = 0.050$), IL-6 ($P = 0.021$), and IIT ($P = 0.005$; Figure 1). Stimulation with IL-1β and IIT caused a significant ($P = 0.039$ and $P = 0.050$, respectively) decrease in CD-RAP mRNA expression in chondrocytes, whereas other cytokine treatments did not result in significant changes (Figure 2).

In FLSs, expression of SAA mRNA was also increased significantly over time in response to stimulation with IL-1β ($P = 0.033$), TNF-α ($P = 0.009$), IL-6 ($P = 0.002$), and IIT ($P = 0.006$; Figure 3). Culture with IIT induced significant increases in MMP-1 and MMP-3 mRNA expression ($P = 0.033$ and $0.050$, respectively), and MMP-3 mRNA in unstimulated control cultures over time were also not significant.

In chondrocytes, expression of SAA mRNA increased significantly over time in response to stimulation with IL-1β ($P = 0.001$), TNF-α ($P = 0.050$), IL-6 ($P = 0.021$), and IIT ($P = 0.005$; Figure 1). Stimulation with IL-1β and IIT caused a significant ($P = 0.039$ and $P = 0.050$, respectively) decrease in CD-RAP mRNA expression in chondrocytes, whereas other cytokine treatments did not result in significant changes (Figure 2).

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Serum amyloid A stimulation of chondrocytes and FLSs

In chondrocytes, exposure to recombinant human SAA resulted in significantly increased expression of SAA (P = 0.005), IL-6 (P = 0.004), MMP-1 (P = 0.001), and MMP-3 (P = 0.014) mRNA over time (Figure 6). Expression of CD-RAP mRNA decreased significantly (P < 0.001) in SAA-treated cells. Stimulation with IL-1β, used as a positive control in this experiment, caused similar significant changes in expression of all target genes (P < 0.05 for all comparisons). In the unstimulated control chondrocyte cultures, SAA expression decreased significantly (P = 0.019) and CD-RAP expression increased significantly (P = 0.038) over time. Changes in expression of IL-6, MMP-1, and MMP-3 mRNA were nonsignificant in control cultures.

In FLSs, SAA treatment significantly increased expression of SAA (P = 0.014), IL-6 (P = 0.004), and MMP-3 (P = 0.006) mRNA over time, whereas the change in MMP-1 expression was nonsignificant (Figure 7). Expression of mRNA for these 4 cytokines in the unstimulated controls did not change significantly over time. Stimulation of equine FLSs with IL-1β, used as a positive control, caused (P < 0.05 for all comparisons).

Discussion

In the present study, equine chondrocytes and FLSs had significantly increased expression of SAA mRNA over time when stimulated by proinflammatory cytokines for ≤ 48 hours in vitro. The SAA mRNA was translated into protein, which was detected in lysed cells and was also released into the culture medium as shown by immunoturbidimetry and isoelectric focusing western blot techniques. These findings corroborate and add to the results of previous studies that revealed expression of SAA in chondrocytes of rabbits with experimentally induced arthritis, in cultured chick embryo chondrocytes stimulated with lipopolysaccharide or IL-6,24 and in synovial tissues derived from inflamed joints of rabbits and people.9,12,14,25,26 In another study, a 4-fold increase in SAA mRNA expression in isolated equine articular chondrocytes stimulated with IL-1β for 6 hours was identified by use of cDNA array techniques. However, as those cells had been passaged 5 to 7 times, it is not clear whether they had retained the chondrocyte phenotype at the time of cytokine stimulation. It has been shown previously that chondrocytes can revert to a more fibroblast-like phenotype when kept in monolayer culture for an extended period of time28 and that this change is accompanied by increased expression of SAA mRNA after 9 days of culture.28 It is therefore important that chondrocytes have had only brief exposure to monolayer culture conditions when aiming to study SAA production and responses in vitro. In the present study, expression of the chondrogenic marker gene CD-RAP remained stable or increased slightly, whereas changes following IL-1β, TNF-α, and IL-6 treatment were nonsignificant (Figure 4).

Serum amyloid A protein was detected by immunoturbidimetry of lysed cytokine-stimulated chondrocytes and FLSs and in cell culture medium from cytokine-stimulated chondrocytes regardless of the cytokine protocol used. None of the unstimulated control cultures contained detectable amounts of SAA protein. Concentrations of SAA ranged from below the detection limit (0.48 mg/L) to 2.6 mg/L in all samples from cytokine-treated chondrocytes and FLSs in the study. Denaturing isoelectric focusing and western blotting techniques allowed detection of several major SAA isoforms in lysed cytokine-stimulated chondrocytes and their culture medium (Figure 5).

Figure 5—Composite image of selected representative western blots depicting isoforms of SAA in equine chondrocyte supernatants after 48 hours of exposure to recombinant equine TNF-α (50 ng/mL) or with IIT (20 ng of each cytokine/mL), respectively. Columns 3, 4, and 5 show SAA isoforms detected in synovial fluid (column 6) and serum (column 7) obtained from horses with lipopolysaccharide-induced synovitis in a previous study2 are shown. Values on the right indicate the measured and extrapolated isoelectric points of the polypeptides assessed from a known marker as described previously. LPS = Lipopolysaccharide. Images in columns 6 and 7 are reproduced from Jacobsen S, Niewold TA, Thomsen MH, et al. Serum amyloid A isoforms in serum and synovial fluid in horses with lipopolysaccharide-induced arthritis. Vet Immunol Immunopathol 2006;10:325–330, (Reprinted from Vet Immunol Immunopathol 2006;10:325–330, with permission from Elsevier.)
cytes did not dedifferentiate or attain an inflammatory phenotype as a result of the culture system.

The cytokine signaling involved in the pathogenesis of joint disease is very intricate, and studying effects of single cytokines or simple cytokine combinations does not reflect the complex interactions of all bioactive compounds involved in the disease process. It is, however, necessary to study individual effects before more complex relationships can be understood, and in the present study IL-1β and TNF-α were identified as potent inducers of SAA synthesis in equine chondrocytes and FLSs. Although some controversy exists, it is generally accepted that these 2 cytokines have central roles in the pathogenesis of osteoarthritis through direct catabolic effects on articular cartilage as well as upregulation of several inflammatory mediators.29

Although the roles of IL-1β and TNF-α in the pathogenesis of joint disease are well established,30 knowledge regarding the involvement of IL-6 is much more incomplete. In rabbit synoviocytes, IL-6 has been shown to induce SAA expression31; however, to the authors’ knowledge, the present study was the first to investigate effects of IL-6 on SAA expression in equine articular tissues. It has been suggested that IL-6 has indirect regulatory or immunomodulatory roles intra-articularly because it seems to exert its effects through influence on responses mediated by IL-1β, TNF-α, and other cytokines and growth factors.29 Interleukin-6 is induced in inflamed synovial tissue, and treatment with soluble IL-6 receptor has been shown to ameliorate rheumatoid arthritis in people.32 Interleukin-6 is also produced by equine chondrocytes and synoviocytes stimulated with lipopolysaccharide in vitro,33 and horses with osteochondral fragmentation associated with carpal chip fractures or osteoarthritis have been shown to have very high concentrations of IL-6 in synovial fluid and IL-6 expression in the fragment.34,35 Together, these findings suggest that IL-6 is involved in the pathogenesis of joint disease in horses and other species.

The SAA isoforms found in lysed cytokine-stimulated articular chondrocytes and in their culture medium in the present study corresponded to those previously identified in synovial fluid from horses with arthritis.1,3 The 3 SAA isoforms with apparent isoelectric point values of 7.9, 8.6, and 9.6 are present not only in synovial fluid but also in serum of horses with arthritis, and we therefore previously suggested that these isoforms were produced in the liver and gained access to the synovial fluid from the bloodstream through the increased permeable synovial membrane of the inflamed joint.1,3 However, the results of the present study showed that these isoforms were also present intracellularly in cytokine-stimulated chondrocytes and that they were released from cells into the culture medium. They are thus not synthesized by hepatocytes alone, and their presence in synovial fluid may result from local synthesis as well as insudation from blood. The isoform with an apparent isoelectric point of 10.2 appears to be synthesized only in extrahepatic cell types because it was detected in cytokine-stimulated chondrocytes and their culture medium in the present study and was found in synovial fluid from a horse with lipopolysaccharide-induced synovitis in another study,3 but was absent from serum of the same affected horse. Although not yet fully elucidated, functions of the different SAA isoforms may vary. Studies have suggested that SAA3 might have antibacterial functions36,37 or serve as a functional mediator of connective tissue metabolism in inflammatory conditions through its binding to fibroblasts.38 While SAA mRNA expression was induced in FLSs as well as chondrocytes in response to stimulation with proinflammatory cytokines in the present study, SAA protein was detected much more consistently in chondrocytes (which had detectable protein identified by immunoturbidimetry and dena-
The phylogenetic conservation of SAA and synthesis of the protein in large quantities in response to injury and infection inspired the hypothesis that the SAA protein has important roles in the host response during inflammatory conditions. There is growing evidence that SAA is directly involved in several mechanisms central to the pathogenesis of inflammatory joint disease.13,16,26,41

In the present study, exposure of equine articular chondrocytes and FLSs to recombinant human SAA caused an increase in gene expression of SAA, IL-6, MMP-1 and MMP-3. Recent studies using FLSs from the synovial membrane of human patients with rheumatoid arthritis showed that SAA is a potent inducer of IL-6 production.40,41 The results of the present study corroborate and expand on those findings by demonstrating that chondrocytes as well as FLSs respond with increased IL-6 expression after being exposed to SAA in vitro.

By revealing the upregulation of MMP-3 gene in equine FLSs exposed to SAA, the results of the present study corroborate the results of previous studies13,16,42 of humans and laboratory animals that indicated SAA is a potent inducer of MMPs in articular chondrocytes and FLSs. Matrix metalloproteinases are involved in degradation of cartilage extracellular matrix, and SAA may thus contribute to destruction of articular cartilage in horses with joint inflammation.

Chondrocyte metabolism was assessed in this study by analysis of CD-RAP gene expression. The CD-RAP protein, which is produced by and secreted from chondrocytes, is considered anabolic owing to its presence in high concentrations in developing cartilage.15,44 Although its functions are far from clear, it has been suggested that CD-RAP is involved in maintaining integrity of the articular cartilage.44 Concentrations of CD-RAP are negatively correlated with degree of cartilage degradation and severity of intraarticular inflammation, as chondrocytes in inflamed joints seem to lose their capacity for CD-RAP synthesis. Intraarticular inflammation induced by injection of lipopolysaccharide has been shown to result in decreased concentrations of CD-RAP in synovial fluid of horses.45 The decrease in CD-RAP expression in equine chondrocytes after culture with SAA (as well as IL-1β or IIT) in the present study thus further suggests that SAA may contribute to alterations in chondrocyte metabolism with potential relevance for the pathogenesis of joint disease.

In horses, very little information is available on functions of SAA, but its rapid induction during inflammation,46 extrahepatic expression at inflammatory sites,10,12,25 and ability to induce proinflammatory processes suggest that SAA may be involved in the pathogenesis of inflammatory joint disease in horses, similar to what has recently been suggested in humans.26 As suggested by others,47,48 these findings seem to indicate that horses may be of value in the study of disease processes involved in human articular inflammation. Because of a pronounced SAA response (with serum concentrations of 10 mg/L or more in horses with systemic infection), horses are particularly well-suited for studies of the SAA response. Our results indicated that SAA may contribute to the pathogenesis of equine inflammatory joint disease and to pathological changes such as cartilage degradation within joints.
Footnotes
a. Gibco, Paisley, Scotland.
b. Eli Lilly, Ballerup, Denmark.
c. Sigma-Aldrich, Steinheim, Germany.
d. Roche Diagnostics, Mannheim, Germany.
e. MP Biomedicals, Solon, Ohio.
h. RNeasy Plus Mini Kit, QiAGEN, Hilden, Germany.
i. Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, Calif.
j. LightCycler 480 SYBR Green I Master mix, Roche Diagnostics, Mannheim, Germany.
k. LightCycler 480 Real-Time PCR System, Roche Diagnostics, Mannheim, Germany.
o. Amersham Pharmacia Biotech, Uppsala, Sweden.
q. Tridelta Development Ltd, Kildare, Ireland.

References


Appendix

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Genbank accession No. or primer source</th>
</tr>
</thead>
</table>
| SAA      | Forward: CCT GGG CTG CTA AAG TCA TC  
           | Reverse: AGG CCA TGA GGT CTG AAG TG     | AF240364.1                           |
| IL-6     | Forward: ATG GCA GAA AAA GAG GGA TG  
           | Reverse: GGG TCA TCA GGG GTG GTT ACT TC | Haneda et al*2009                    |
| MMP-1    | Forward: CAG TGC CTT CAG AAA CAC GA  
           | Reverse: GCT TCC CAG TCA CTT TCA GC    | AF148882.1                           |
| MMP-3    | Forward: TGT GGA GGT GAT GCA CAA ATC 
           | Reverse: GCA TGC CAG GAA ATG TAG TGA A | NM_001082495.2                       |
| CD-RAP*  | Forward: ATG CCC AAG CTG GCT GA     | EF79787                               |
| GAPDH    | Forward: AGG TGG TGG ACC AAG GGT CAT CAT 
           | Reverse: AGC TTT CTC CAG GCG GCA GGT CAG | Iqbal et al*2004                     |

*Primer were designed by the authors unless otherwise indicated.
*Also identified as the Melanoma Inhibitory Activity gene.