CHAPTER 4

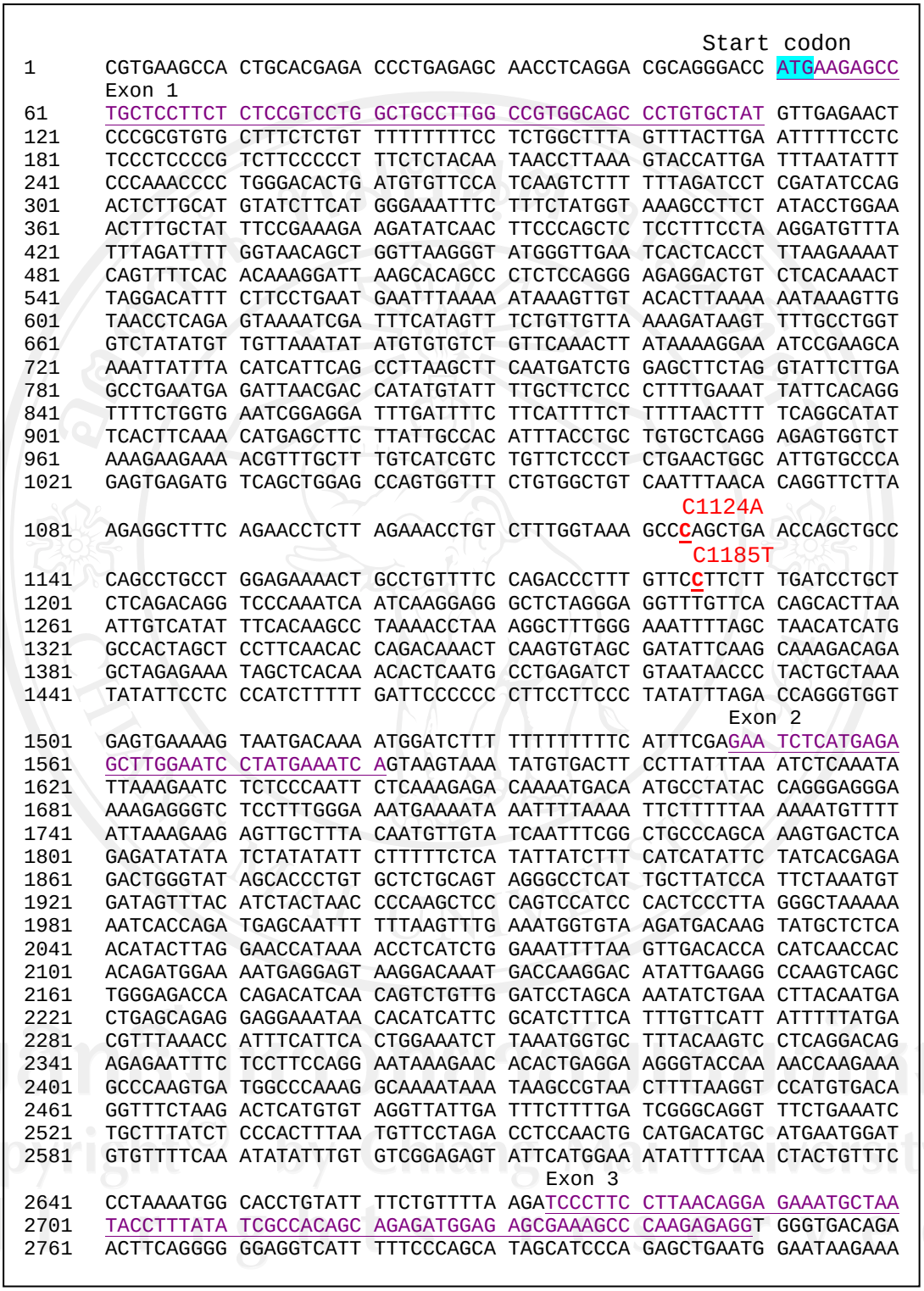
RESULTS AND DISCUSSION

4.1 Characterization of porcine *MGP* gene

The nucleotide sequence of porcine *MGP* gene was determined by genomic DNA sequencing. The partial sequence of *MGP* gene (Figure 4.1), spans 4060 bp of genomic DNA from the start site of transcription and contains four exons (Genbank Accession no. NC010447.1) separated by three large intervening sequences. There comprise a 5' untranslated region (5' UTR) of 50 bp followed by coding region for 103 amino acids from the start codon (ATG) to the stop codon (TAA) and 226 bp of 3' UTR. Exons are ranging in length from 33 to 287 bp. All the exons-introns boundaries have the consensus GT-AG splicing sequences. For the last exon, the transcription termination is shown in bold italics instead of a splice donor (Table 4.1).

Table 4.1 Exon-intron junctions of the porcine *MGP* gene

Exon	Exon size (bp)	Splicing donor	Intron size (bp)	Splicing acceptor
1	60	CCTGTGCTAT**gt**tgag	1437	ttcg**ag**AATCTCATGA
2	33	TATGAAATCA**gt**aagt	1091	ttaa**ag**ATCCCTTCCT
3	78	CCCAAGAGAG**gt**gggt	972	ttct**ag**AATCCGAGAA
4	287	GGCCAAATAA***G***accag		

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                                                                Start codon
1       CGTGAAGCCA CTGCACGAGA CCCTGAGAGC AACCTCAGGA CGCAGGGACC ATGAAGAGCC
        Exon 1
61      TGCTCCTTCT CTCCGTCCTG GCTGCCTTGG CCGTGGCAGC CCTGTGCTAT GTTGAGAACT
121     CCCGCGTGTG CTTTCTCTGT TTTTTTTTCC TCTGGCTTTA GTTTACTTGA ATTTTTCCTC
181     TCCCTCCCCG TCTTCCCCCT TTCTCTACAA TAACCTTAAA GTACCATTGA TTTAATATTT
241     CCCAAACCCC TGGGACACTG ATGTGTTCCA TCAAGTCTTT TTTAGATCCT CGATATCCAG
301     ACTCTTGCAT GTATCTTCAT GGGAAATTTC TTTCTATGGT AAAGCCTTCT ATACCTGGAA
361     ACTTTGCTAT TTCCGAAAGA AGATATCAAC TTCCCAGCTC TCCTTTCCTA AGGATGTTTA
421     TTTAGATTTT GGTAACAGCT GGTTAAGGGT ATGGGTTGAA TCACTCACCT TTAAGAAAAT
481     CAGTTTTCAC ACAAAGGATT AAGCACAGCC CTCTCCAGGG AGAGGACTGT CTCACAAACT
541     TAGGACATTT CTTCCTGAAT GAATTTAAAA ATAAAGTTGT ACACTTAAAA AATAAAGTTG
601     TAACCTCAGA GTAAAATCGA TTTCATAGTT TCTGTTGTTA AAAGATAAGT TTTGCCTGGT
661     GTCTATATGT TGTTAAATAT ATGTGTGTCT GTTCAAACTT ATAAAAGGAA ATCCGAAGCA
721     AAATTATTTA CATCATTCAG CCTTAAGCTT CAATGATCTG GAGCTTCTAG GTATTCTTGA
781     GCCTGAATGA GATTAACGAC CATATGTATT TTGCTTCTCC CTTTTGAAAT TATTCACAGG
841     TTTTCTGGTG AATCGGAGGA TTTGATTTTC TTCATTTTCT TTTTAACTTT TCAGGCATAT
901     TCACTTCAAA CATGAGCTTC TTATTGCCAC ATTTACCTGC TGTGCTCAGG AGAGTGGTCT
961     AAAGAAGAAA ACGTTTGCTT TGTCATCGTC TGTTCTCCCT CTGAACTGGC ATTGTGCCCA
1021    GAGTGAGATG TCAGCTGGAG CCAGTGGTTT CTGTGGCTGT CAATTTAACA CAGGTTCTTA
                                                    C1124A
1081    AGAGGCTTTC AGAACCTCTT AGAAACCTGT CTTTGGTAAA GCCCAGCTGA ACCAGCTGCC
                                                     C1185T
1141    CAGCCTGCCT GGAGAAAACT GCCTGTTTTC CAGACCCTTT GTTCCTTCTT TGATCCTGCT
1201    CTCAGACAGG TCCCAAATCA ATCAAGGAGG GCTCTAGGGA GGTTTGTTCA CAGCACTTAA
1261    ATTGTCATAT TTCACAAGCC TAAAACCTAA AGGCTTTGGG AAATTTTAGC TAACATCATG
1321    GCCACTAGCT CCTTCAACAC CAGACAAACT CAAGTGTAGC GATATTCAAG CAAAGACAGA
1381    GCTAGAGAAA TAGCTCACAA ACACTCAATG CCTGAGATCT GTAATAACCC TACTGCTAAA
1441    TATATTCCTC CCATCTTTTT GATTCCCCCC CTTCCTTCCC TATATTTAGA CCAGGGTGGT
                                                              Exon 2
1501    GAGTGAAAAG TAATGACAAA ATGGATCTTT TTTTTTTTTC ATTTCGAGAA TCTCATGAGA
1561    GCTTGGAATC CTATGAAATC AGTAAGTAAA TATGTGACTT CCTTATTTAA ATCTCAAATA
1621    TTAAAGAATC TCTCCCAATT CTCAAAGAGA CAAAATGACA ATGCCTATAC CAGGGAGGGA
1681    AAAGAGGGTC TCCTTTGGGA AATGAAAATA AATTTTAAAA TTCTTTTTAA AAAATGTTTT
1741    ATTAAAGAAG AGTTGCTTTA CAATGTTGTA TCAATTTCGG CTGCCCAGCA AAGTGACTCA
1801    GAGATATATA TCTATATATT CTTTTTCTCA TATTATCTTT CATCATATTC TATCACGAGA
1861    GACTGGGTAT AGCACCCTGT GCTCTGCAGT AGGGCCTCAT TGCTTATCCA TTCTAAATGT
1921    GATAGTTTAC ATCTACTAAC CCCAAGCTCC CAGTCCATCC CACTCCCTTA GGGCTAAAAA
1981    AATCACCAGA TGAGCAATTT TTTAAGTTTG AAATGGTGTA AGATGACAAG TATGCTCTCA
2041    ACATACTTAG GAACCATAAA ACCTCATCTG GAAATTTTAA GTTGACACCA CATCAACCAC
2101    ACAGATGGAA AATGAGGAGT AAGGACAAAT GACCAAGGAC ATATTGAAGG CCAAGTCAGC
2161    TGGGAGACCA CAGACATCAA CAGTCTGTTG GATCCTAGCA AATATCTGAA CTTACAATGA
2221    CTGAGCAGAG GAGGAAATAA CACATCATTC GCATCTTTCA TTTGTTCATT ATTTTTATGA
2281    CGTTTAAACC ATTTCATTCA CTGGAAATCT TAAATGGTGC TTTACAAGTC CTCAGGACAG
2341    AGAGAATTTC TCCTTCCAGG AATAAAGAAC ACACTGAGGA AGGGTACCAA AACCAAGAAA
2401    GCCCAAGTGA TGGCCCAAAG GCAAAATAAA TAAGCCGTAA CTTTTAAGGT CCATGTGACA
2461    GGTTTCTAAG ACTCATGTGT AGGTTATTGA TTTCTTTTGA TCGGGCAGGT TTCTGAAATC
2521    TGCTTTATCT CCCACTTTAA TGTTCCTAGA CCTCCAACTG CATGACATGC ATGAATGGAT
2581    GTGTTTTCAA ATATATTTGT GTCGGAGAGT ATTCATGGAA ATATTTTCAA CTACTGTTTC
                                                  Exon 3
2641    CCTAAAATGG CACCTGTATT TTCTGTTTTA AGATCCCTTC CTTAACAGGA GAAATGCTAA
2701    TACCTTTATA TCGCCACAGC AGAGATGGAG AGCGAAAGCC CAAGAGAGGT GGGTGACAGA
2761    ACTTCAGGGG GGAGGTCATT TTTCCCAGCA TAGCATCCCA GAGCTGAATG GAATAAGAAA
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Figure 4.1 Nucleotide sequences of porcine *MGP* gene with 4060 bp genomic DNA, the start and stop codon and polymorphisms position.

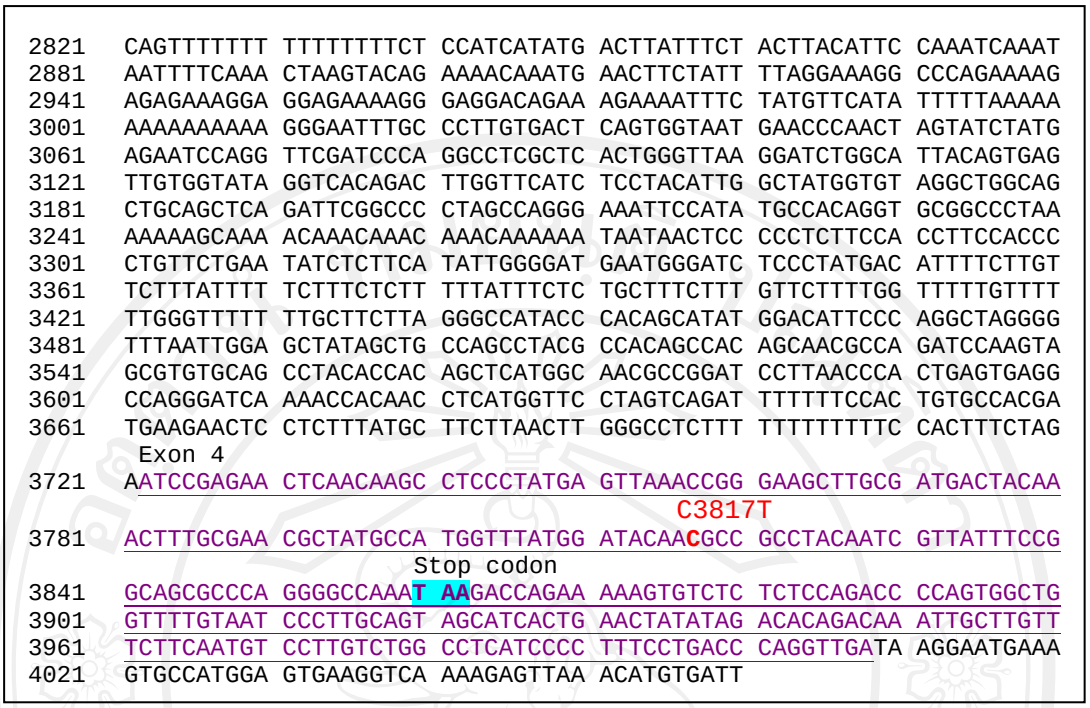

Figure 4.1 Nucleotide sequences of porcine *MGP* gene with 4060 bp genomic DNA, the start and stop codon and polymorphisms position (continued).

Sequence analysis of the genomic region upstream of the putative transcription start site indicated the absence of TATA and CCAAT boxes. The translation start codon was assigned based on the homology to the human ortholog. The polyadenylation signal AATAAA is located approximately 205 bp downstream of the stop codon resulting in the DNA sequence of porcine *MGP* gene (Genbank Accession no: NC010447) approximately 4088 bp. The porcine *MGP* amino acid sequence shows identities of 85, 92, 87 and 84% to human (Genbank Accession no: NP000891), bovine (Genbank Accession no: NP777132), mouse (Genbank Accession no: NP032623) and rat (Genbank Accession no: NP036994), respectively. Comparison between porcine and mammalian *MGP* genes indicates that all introns are located at conserved sites within the human and bovine *MGP* coding sequence (Figure 4.2). Moreover, the characteristic motifs previously identified in other mammalian *MGP* are also present in the porcine protein. These include a highly conserved domain at the N-terminus which may be subject to serine phosphorylation (motif SxESxESxE) and five glutamic acid residues which are usually modified by γ-

carboxylase that are carboxylated in a vitamin K dependent process (Price *et al*., 1994). The fact that this site is so highly conserved suggests that it is required for the correct regulation of *MGP* function (Cancela *et al*., 2001).

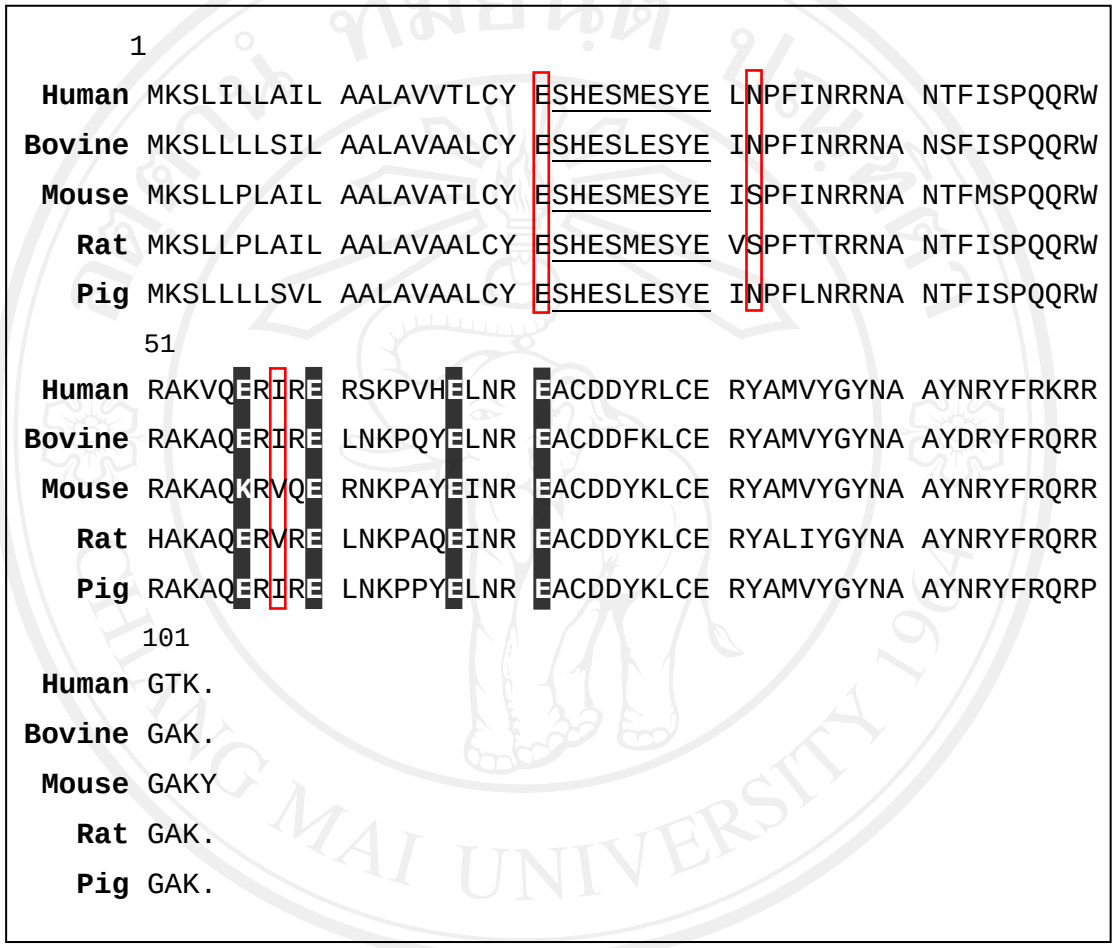

Figure 4.2 Multiple alignment of different mammalian *MGP* amino acid sequences. Conserved sites of intron insertions in porcine and mammalian *MGP* are boxed. The γ-carboxyglutamate residues are shown in black boxes. Motifs previously identified in mammalian *MGP* are underlined.

4.2 Identification of polymorphisms in porcine *MGP* gene

The search for sequence variations by comparative sequencing within the *MGP* gene revealed a total of 3 SNPs shown in figure 4.1. The PCR fragments were amplified with primers (Table 3.3) that covers the polymorphic sites. Two SNPs were located in intron 1, a transversion from cytosine (C) to adenine (A) at position 1124

and transition from cytosine (C) to thymine (T) at 1185 (Figure 4.3). Another SNP at position 3817 in exon 4 was transition from cytosine (C) to thymine (T). These SNP was a synonymous substitution on amino acid of porcine *MGP* gene.

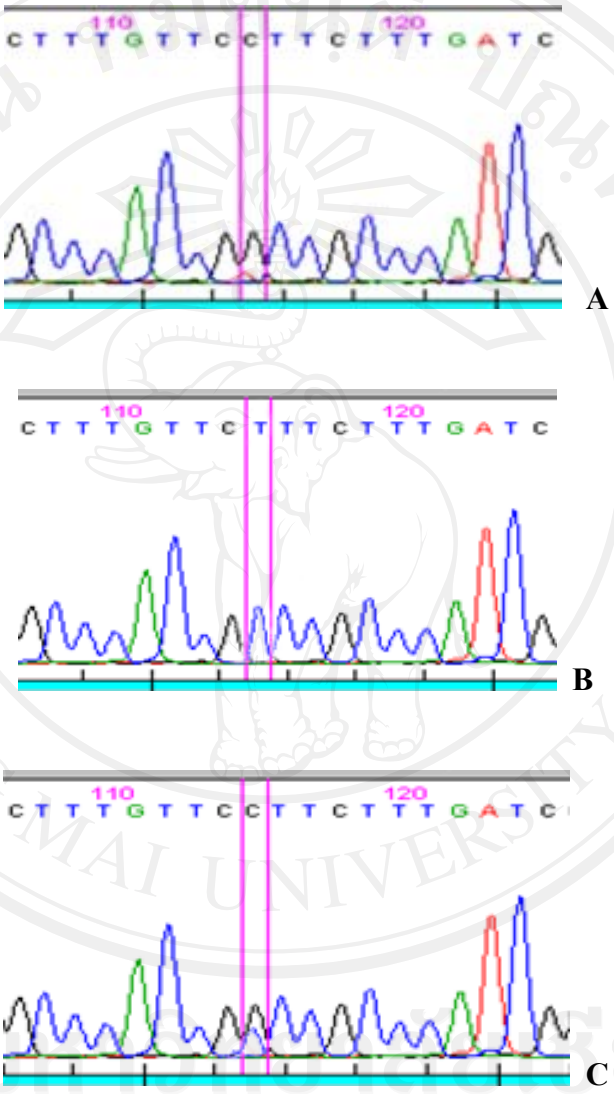

Figure 4.3 A representative image showing SNP by comparative sequencing. Column show SNP position. A) sequence with homozygous T, B) sequence with homozygous C, and C) sequence with heterozygote TC.

4.3 Expression profile of *MGP* gene in articular cartilage

In this study, RT-PCR analysis indicated that the porcine *MGP* gene was shown no difference (P>0.05) between healthy and OC lesions samples of articular cartilage (Figure 4.4). *MGP* was presented in all articular cartilages tested and was shown the lowest level in muscle. Quantitative real time PCR results showed no differentiation levels of expression in both groups. It should be noted that the expression of *MGP* gene also found no differences in healthy and OC pigs, this may due to a number of samples, only 12 pigs, were examined in this experiment. However, other studies have found evidence of *MGP* expression in proliferative and late hypertrophic chondrocytes in chondrogenic *in vitro* model. *MGP* overexpression delayed chondrocyte maturation and blocked endochondral ossification of cartilage (Yagami *et al*., 1999; Newman *et al*., 2001). Several features in *MGP* deficiency suggest involvement of vascular endothelium that may result from a failure in angiogenesis or vessel fusion and arterial calcification (Lou *et at*., 1997; Munroe *et al*., 1999). These may relate to endothelial dysfunction during vascular maturation and loss of architecture and hypertrophic chondrocytes in the bone growth plate that may relate to disturbed vascularization (Warburton *et al*., 2000).

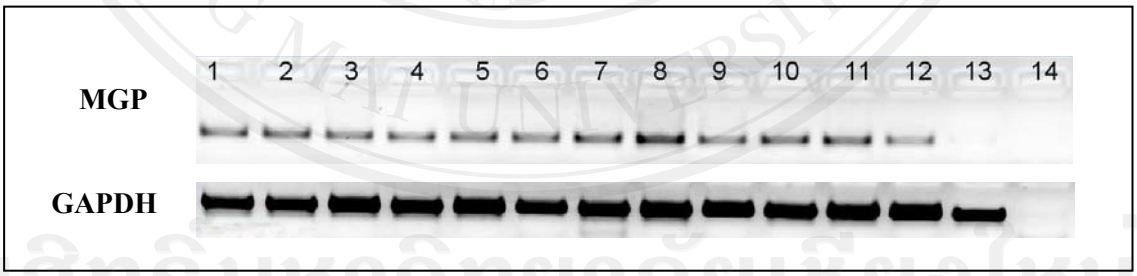

Figure 4.4 Relative levels of *MGP* gene expression in total mRNA from healthy (lane 1-6) and OC lesions (lane 7-12) articular cartilage in pig. Lane 13 is RNA from meat and lane 14 is negative control reactions.

4.4 Identification of polymorphisms in candidate genes

Polymorphisms in candidate genes were detected in pool DNA from 8 individual experimental animals by comparative sequencing. Primers were designed from corresponding gene sequence and used to amplify overlapping fragments covering exon and intron parts. The sequences of DNA fragments were compared using the BLAST software. In order to confirm the polymorphisms each individual animals in pool DNA were sequenced that revealed a potential polymorphic site after the first analysis.

4.4.1 Polymorphism in the porcine *TGFβ1*

Primers were designed from the published *TGFβ1* gene sequence (GenBank Accession no: AF461809 and AF461808) and used to amplify overlapping fragment of about 218 and 1083 bp in size covering part of exon 5 – intron 6. One polymorphism was a transition from Adenine (A) to Guanine (G) within exon 5 at position 797 of coding sequence. A forward primer located in intron 4 (Figure 4.5) and a reverse primer position located in exon 5 were derived to produce a 218 bp PCR product from porcine genomic DNA. Theses SNP has no effect on amino acid sequence of porcine *TGFβ1*. Using PCR with primers encompassing the intron 6 (Figure 4.6), sequence comparison of these fragment was found two SNPs. First, a transition from adenine to guanine (A > G) at position 179, another transition (C > T) was found at position 1042 of the intron 6.

```
  1 TGAGGCCCGC CTCCCTGGCC CAGCCCTGTG CCCAGCAGTG ACTCTGTGCG
 51 TGTGTGTGCA CACGGCGTGC GCACGTGCGC GCGGTGGGCG GGTTCTTCCC
                 TGFβ1 F1
101 CTGCCCACCC CCTACTCATC CATCTGAGTG TGTGTGTGTA TGTCTCCCCC
151 AACCCTATCC GCTCCCTGAC TCGTAAACCA AAGCAG
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Figure 4.5 Sequence of intron 4 of *TGFβ1*gene (Accession number AF461809).

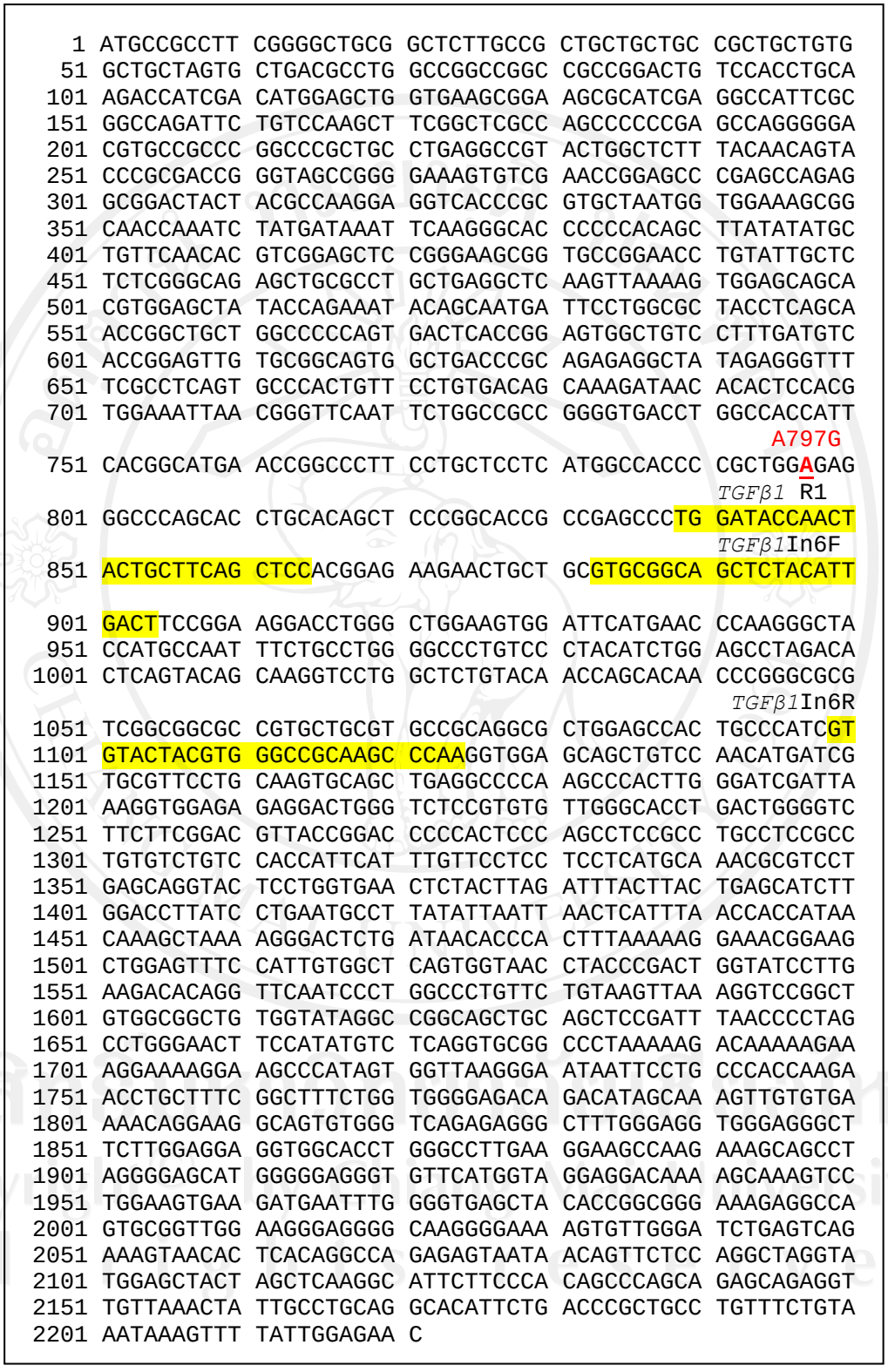

Figure 4.6 Nucleotide sequence of *TGFβ1* gene mRNA (Accession number: AF461808).

4.4.2 Polymorphism in the porcine *MMP3*

One polymorphism was found within porcine *MMP3* gene. The *MMP3* primer formed a 430 bp long amplicon (spanning intron 2) in pig (GenBank Accession no: AB044413). The SNP was a transition from C to T at position 158 of amplified fragment in intron 2.

Figure 4.7 *MMP3* sequence, showing primer sequences and polymorphism in intron 2.

4.4.3 Polymorphism in the porcine *COL2A1*

Primers derived from the published *COL2A1* gene sequence (GenBank Accession no: AF201724) were used to amplified. The PCR fragments of 397 bp were amplified with primer (Table 3.3) that covers two polymorphic sites at position 81 ('C/T' transition) and 156 ('G/A' transition) in intron 29 of porcine *COL2A1* (Figure 4.8).

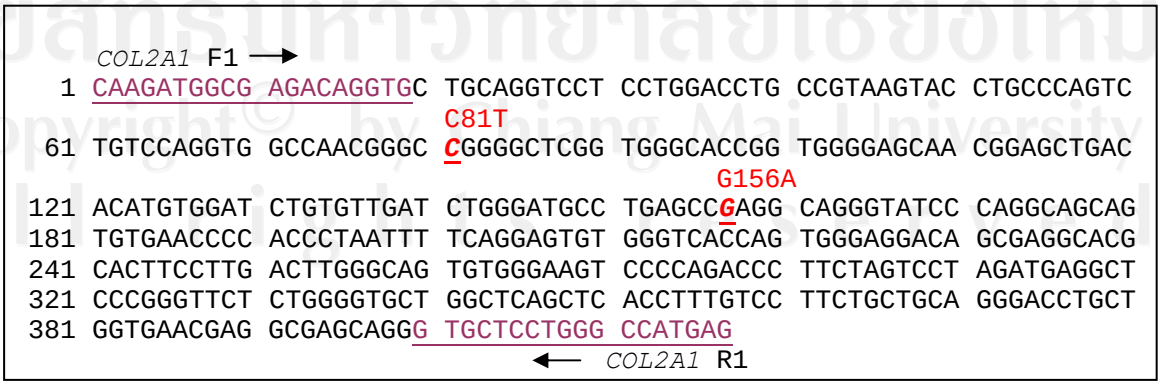

Figure 4.8 *COL2A1* sequence, showing primer sequences and polymorphism in intron 29.

4.4.4 Polymorphism in the porcine *COL10A1*

Screening for polymorphisms in porcine *COL10A1* gene revealed one SNP at position 103 in exon 1. The SNP was a transition from A to G (Figure 4.9). The comparison of amino acid sequences derived for the sequence revealed that the polymorphism has no effect.

```
    COL2A1 F4 ⟶
  1 TTCAGCCTAC CTCCATATGC ATTGTGTGGG GCGGGGTGGA GCTGGGGAAC ACCTTTTCAT
 61 GCAGATTTAT ATTAGCTGGG CAGGAATGGG CTCACATTGG AGCCACTAGG AATCCTGAGA
121 AAGAGGAGTG GACGTACTCA GAGGAGTACA GCCCGTTCGA CCCAGCGTTG GGCAGCTGGA
                A103G
181 GCCATACCTG GTCATTATCT GTGAGATCAA GGATGGCACT CCCTGAAGCC TGATCCAGGT
241 AGCCTTTGAC ATATTCATCA TAGGTGTACA TGACAGGGGT GCCATTTTTA TACAGGCCCA
321 CCCAAGCATG GGTCCCCTTC ACGTGAATGT GGTAGGAGAA GTAATTGATT CCAGGTATCC
381 TGCAGGTAAA GATTCCAGTT TTTGGGTCGT AGTGCTGTTG
                                  ⟵ COL2A1 R4
```

Figure 4.9 *COL10A1* sequence, showing primer sequences and polymorphism in exon 1.

4.5 Development of multiplex SNP genotyping

In total of 10 SNPs were identified in this study. The multiplex SNP were established to facilitate large scale genotyping by using SNP primer extension technique. The primer anneals one base short of the target SNP. The length of the gene-complementary portion of the primer is selected to give a Tm of 69°– 75°C. The genomic DNA containing the SNPs were amplified by multiplexed PCR and used as template for genotyping. Genomic DNA was amplified to multiplex PCR in the presence of 7 primers pairs (Table 3.3) and confirmed each fragment by individual PCR (Figures 4.10). Primer extension step was attempted via the addition of labelled ddNTPs (CEQ SNP-Primer Extension Kit, Beckman Coulter) to SNP-specific oligonucleotide primers, prior to allele identification by analysis on a DNA sequencer (Beckmann Coulter CEQ8000) (Figure 4.11). The SNP primers extension included in the speciation assay range in size from 21 bp to 79 bp, although migration does not exactly correspond to primer size (Table 3.5). SNP primers were all separated by a migration gap of at least 4 bp to ensure ease of identification. As recommended by Beckman, the annealing temperature used for the generation of the primer extension

product at 64°C was selected to be at least 5°C below the lowest primer midpoint temperature.

All SNPs were genotyped in 310 animals of the F2-DUPI resource population including the animals of the grandparental generation (Duroc and Pietrain pig) and the F1 parental generations (data show in the appendix). Genotype frequencies are shown in Table 4.3. Multiplexed SNP genotyping using the CEQ8000 genetic analysis system was explored further by determining 10 different SNP genotypes for 96 genomic DNA samples. The result demonstrates an efficient process of producing for 960 SNP genotypes in total and was able to call the correct genotypes with high degree of accuracy approximately 96%.

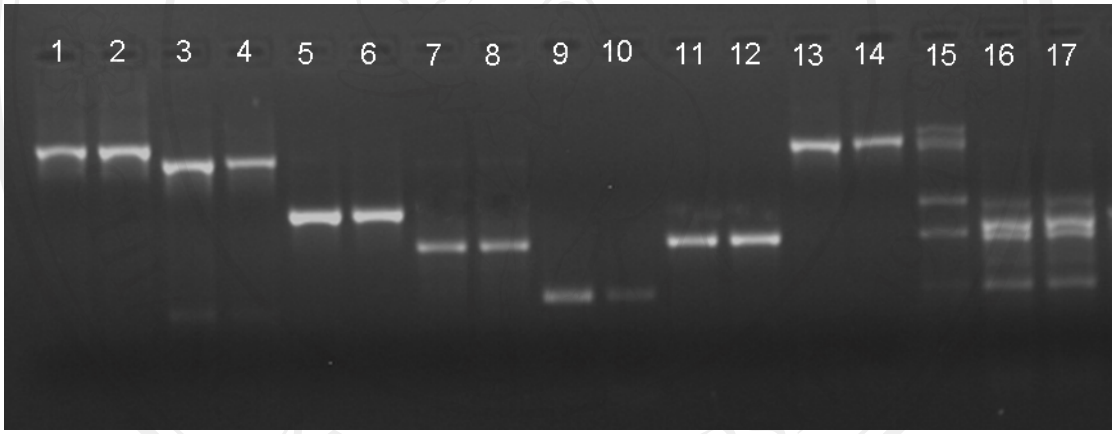

Figure 4.10 Agarose gel analysis of normal PCR and multiplex PCR products after amplification. Lane:1-2, *MGP*WF1/WR1 primer (1088 bp), Lane:3-4, *MGP*F2/R1 primer (1161 bp), Lane:5-6, *MMP3*F1/R1 primer (434 bp), Lane:7-8, *COL2A1*F1/R1 primer (397bp), Lane:9-10, *TGFβ1*F1/R1 primer (218 bp), Lane:11-12, *COL10A1*F4/R4 primer (400 bp), Lane:13-14, *TGFβ1*In6F/R primer (1083 bp), and Lane: 15-17, all primers that used in normal PCR.

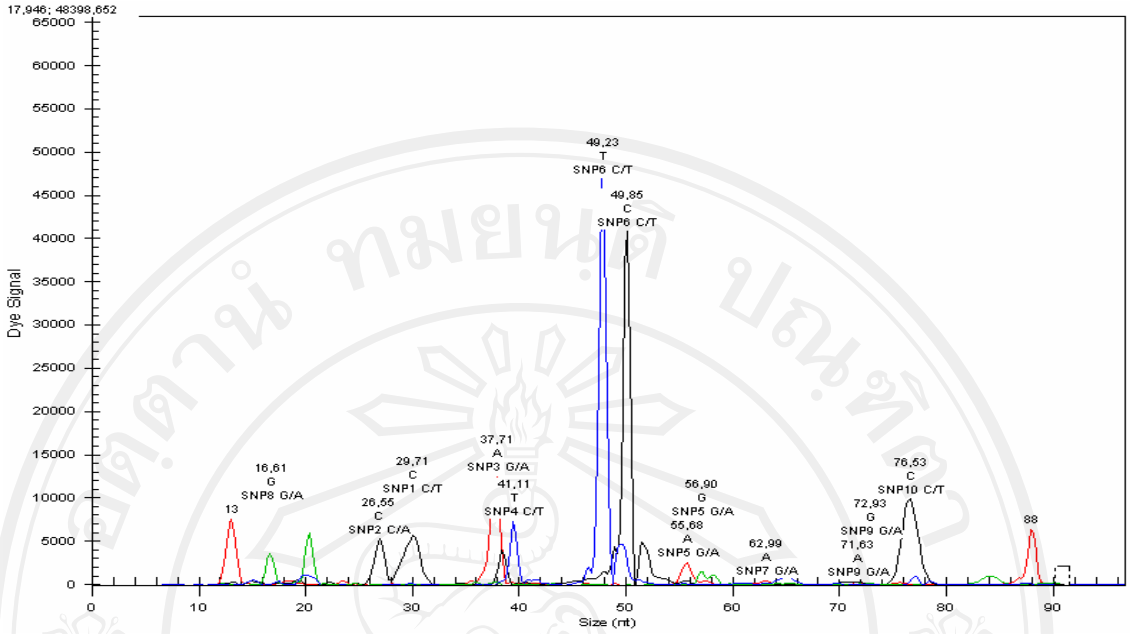

Figure 4.11 Example of the genotype identified by multiplex SNP assay. The first and last peaks represent size standards of 13 and 88 bp, respectively. The ten peaks in assay relate to MGP_{C1124T}, MGP_{C1185A}, MGP_{C3817T}, $MMP3_{C158T}$, $COL2A_{C81T}$, $COL2A1_{G156A}$, $COL10A1_{G103A}$, $TGF\beta1_{A797G}$, $TGF\beta1_B$, $TGF\beta1_C$ moving from the smallest to largest fragment. The colors of the peaks reflect different bases, where T is blue, C is black, A is red and G is green.

4.6 Association analysis

Four of the candidate genes were associated with osteochondrosis and DXA traits, and only one gene (*COL10A1*) was not significantly associated in either trait. Individual polymorphisms in genes *MGP, TGFβ1, MMP3* and *COL2A1* were found to be significantly associated (Table 4.2). Haplotype frequencies were estimated by hbat option of FBAT program. Haplotype TAA and TAG of *MGP* gene were associated with OC as well as DXA traits. Whereas haplotype CA of *COL2A1* was associated with OC in knee joint. However, no association of *TGFβ1* gene and OC and DXA traits were found in this study.

Either of individual polymorphism or haplotype at *MGP* gene was associated with osteochondrosis and DXA traits. This result suggested the function of *MGP* protein on this bone related traits. These results are in agreement with association analysis in human that human *MGP* gene had shown to be significantly associated with BMD trait (Tsukamoto *et al*., 2000, Zebboudj *et al*., 2002). The mechanisms of inhibits mineralization by *MGP* remain unclear. However, at least two mechanisms could account for the inhibitory function of *MGP* on calcification. First, *MGP* is a member of the mineral-binding Gla protein family (Price *et al*., 1998), which includes osteocalcin. *MGP* binds calcium ions and hydroxyapatite via its five γ-carboxylated glutamic acid (Gla) residues. Inhibition of the γ-carboxylation of Gla residues results in increased matrix mineralization, suggesting that the mineral-binding Gla residues are crucial for regulation of matrix mineralization. Second, *MGP* has been shown to modulate the biological activity of the *TGFβ* superfamily members such as bone morphogenetic proteins (*BMPs*) (Boström *et al*., 2004)). It was thereafter reported that *MGP* modulates *BMP* activity in mesenchymal cell differentiation (Price *et al*., 1998). Moreover, deficiency of *MGP* results in multiple developmental defects including abnormal vascularization of the bone growth plate and induced apoptosis, suggesting involvement of a role in cell differentiation (Newmann *et al*., 2001, Zebboudj *et al*., 2004). Another interesting notion, it has been demonstrated that *MGP* protein could be identified in a synovial fluid sample from an osteoarthritis patient but not in a healthy cartilage (Kamphorst *et al.*, 2007).

Table 4.2 Associations of individual SNP with DXA and osteochodrosis lesions

Gene	Allele	Frequencies	*P*-value						
			BMD	BMC	Osteochondrosis lesion				
					Shoulder	Elbow	Hip	Knee	Total
MGPe4	C T	0.36 0.64	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**0.002****	**≤0.001****	**≤0.001****
MGPin1A	G A	0.41 0.59	**0.007****	**0.004****	**0.006****	**0.008****	**0.019***	**0.009****	**0.011***
MMP3in2	G A	0.35 0.65	**≤0.001****	**≤0.001****	**0.003****	**≤0.001****	**0.013***	**0.004****	**≤0.001****
COL2A1in29A	C T	0.39 0.61	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****
COL2A1in29B	G A	0.36 0.64	0.178	0.184	0.280	0.242	0.369	0.538	0.219
TGFB1ex5	C T	0.34 0.66	0.257	0.237	0.089	0.105	0.542	**0.022***	**0.037***
COL10A1e1	G A	0.49 0.51	0.988	0.945	0.466	0.651	0.543	0.569	0.662
MGPin1B	C A	0.47 0.53	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**≤0.001****	**0.007****	**0.001****
TGFB1in6A	G A	0.53 0.47	**0.011***	**0.013***	0.064	**0.013***	**0.006****	**0.022***	**0.006****
TGFB1in6B	C T	0.58 0.42	0.442	0.359	0.48	0.335	0.538	0.831	0.972

ns= no significant ($P>0.05$),* $P\leq0.05$, **$P\leq0.001$

Table 4.3 Estimated haplotype frequencies of *MGP, TGFβ1* and *COL2A1* with DXA and osteochodrosis lesions

Gene, SNP	Haplotype	Frequencies	*P*-value						
			BMD	BMC	Osteochondrosis lesion				
					Shoulder	Elbow	Hip	Knee	Total
***MGP*:**	TAA	0.368	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***
MGPe4,	TAG	0.314	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***	**≤0.01***
MGPin1A,	CAA	0.101	ns	ns	ns	ns	ns	ns	ns
MGPin1B	CCG	0.062	na	na	ns	ns	ns	ns	ns
	CAG	0.061	ns	ns	ns	ns	ns	ns	ns
	TCG	0.047	ns	ns	ns	ns	ns	ns	ns
	TCA	0.047	ns	ns	ns	ns	ns	ns	ns
TGFβ1:	CGC	0.333	ns	ns	ns	ns	ns	ns	ns
TGFB1ex5,	TGC	0.208	ns	ns	ns	ns	ns	ns	ns
TGFB1in6A,	TGT	0.125	ns	ns	ns	ns	ns	ns	ns
TGFB1in6B	TAC	0.104	ns	ns	ns	ns	ns	ns	ns
	CAT	0.083	na	na	na	na	na	na	na
	CAC	0.062	na	na	ns	ns	ns	ns	ns
	CGT	0.042	ns	ns	ns	ns	ns	ns	ns
	TAT	0.042	ns	ns	ns	ns	ns	ns	ns
COL2A1:	TA	0.412	ns	ns	ns	ns	ns	ns	ns
COL2A1in29A,	TG	0.305	ns	ns	ns	ns	ns	ns	ns
COL2A1in29B	CG	0.225	ns	ns	ns	ns	ns	ns	ns
	CA	0.058	ns	ns	ns	ns	ns	**≤0.05***	ns

ns= no significant ($P>0.05$),* $P\leq0.05$, **$P\leq0.001$, na = not available

TGFβ1 promotes type II collagen and proteoglycan synthesis and down regulates cartilage degrading enzymes (Hashimoto *et al*., 2007). *TGFβ1* is secreted by chondrocytes in an inactive form bound to a latency molecule, underlying the importance of defining the factors leading to *TGFβ1* activation in the growth plate (Pedrozo *et al*., 1998). It has been implicated as being responsible for regulator of growth cartilage metabolism, particularly in the control of chondrocytes differentiation and hypertrophy, such as those present during osteoclastic bone resorption (Angelo *et al*., 2001). The result was found significant association in BMD, BMC and OC lesions except lesion on shoulder (P = 0.064) (Table 4.3). In recent years, polymorphisms in *TGFβ1* have been identified and found to be correlated with spine OA in some studies (Horton *et al*., 1997). Furthermore, *in vitro* studies of *TGFβ*1 are a potent inhibitor of maturation, including cell hypertrophy, type-X collagen expression and alkaline phosphatase (ALP) activity (Ballock *et al*., 1993; Schipani *et al*., 1997). ALP is expressed at the cell surface and facilitates the release of inorganic phosphate required for bone formation. ALP activity is elevated in the transitional chondrocytes of chicks with dyschondroplasia, confirming our earlier report (Farquharson *et al.,* 1992c). *TGFβ1* is known to inhibit ALP activity as well as regulate proteoglycan production and collagen synthesis within chondrocytes. *TGFβ1* on other hand, potently enhances the proliferation of osteoprogenesis, but inhibits their terminal differentiation and expression of OC (Ogata *et al*., 1997).

The polymorphism at *MMP3* gene had a very large effect on OC lesions. *MMP3* gene encodes a matrix metalloproteinase involved in articular cartilage extracellular matrix. *MMP*s are responsible for catabolism and turnover of the matrix and are induced during the process of chondrocyte hypertrophy (Angelo *et al*., 2001). Inappropriately high *MMP3* levels would implicate increased protease activity as a cause of abnormal extracellular matrix in cartilage affected by OC (Ohata *et al*., 2002, Kuroki *et al*., 2005). *MMP3* is produced by hypertrophic chondrocytes and is secreted in matrix vesicles. Mineralization of the matrix is associated with a marked increase in the cleavage of type II collagen by *MMPs* (Angelo *et al*., 2001; Mwale *et al*., 2002). Matrix vesicles also contain *TGFβ*, which is present in a latent form but is activated by *MMP3*. The increased levels of active *TGFβ* present in the growth plate at the onset of mineralization are believed to be due, in part, to the presence of *MMPs*

in matrix vesicles. Additionally, *MMPs* are critical for angiogenesis in the growth plate and thus are necessary for normal calcification and bone formation. Mice without *MMPs* have defective angiogenesis, reduced chondrocyte apoptosis, widening of the hypertrophic zone, and decreased mineralization of the matrix (Vu *et al*., 1999). The effects of the *MMPs* on angiogenesis may be related to a decrease in catabolism of the matrix, release of important growth factors, or other effects, but these relationships have not yet been clarified.

Polymorphisms in the *COL2A1* gene have previously been implicated in the risk of OC as well as in the occurrence of chondrocytes. *COL2A1* gene encoding for type II collagen has been shown to cause a variety of chondrodysplasias and osteoarthrosis. It also plays a role in the pathogenesis of disc degeneration (Sahlman, 2007). Mutated type II collagen has been shown to disturb chondrogenesis and decrease bone strength (Savontaus *et al*., 1996). Recent studies of animals have demonstrated that uremia, which retards growth in children, is associated with an increase in the width of hypertrophic cartilage as well as a decrease in the deposition of both type II collagen, alterations in collagen fibril architecture, and defective mineralization (Alvarez *et al*., 2001). The deletions in the mouse *COL2A1* gene result in dysmorphogenesis of the joint leading to poor articulation and biomechanical imbalance in addition to loss of matrix integrity (Helminen *et al.,* 2002). It appears that any mutation in extracellular matrix components that can compromise either matrix integrity or morphogenesis have the potential to predispose a joint to OA.