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Association between mitogen-activated protein kinase 1 (MAP2K1) gene polymorphisms and the risk of adenocarcinoma during aging in bitches*)

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Association between mitogen-activated protein kinase 1 (MAP2K1) gene polymorphisms and the risk of adenocarcinoma during aging in bitches

Summary

Carcinogenesis is often associated with the uncontrolled cell division cycle that leads to the growth and development of cancer. In this study we analyzed the occurrence of MAP2K1 gene polymorphism, which is described as a cell division regulator, in a group of dogs with diagnosed adenocarcinoma and aged group of animals.

In this study, blood samples were obtained from 22 female dogs diagnosed with mammary tumors. Moreover, blood samples from geriatric (> 5 to 10-years-old; n = 15), mature adult (> 2 to 5-years-old; n = 10) and young (from 1 to 2-years-old; n = 11) dogs were also collected. 36 bitches diagnosed because of other reasons served as controls.

After Sanger sequencing analysis, 15 single nucleotide variations were identified, of which 3 were situated in exons (exon 2, 7 and 11) and the remaining 12 were localized in splice regions of introns. We also observed differences in genotype and allele frequencies between tumor and control groups for heterozygote and for an alternative allele for 3 polymorphisms (c.G267T, g.T72562C, g.T75728C). Moreover, we found higher prevalence of the alternative alleles and/or alternative homozygotes solely in the most advanced age subgroup in comparison with the other two subgroups for two pair of variations: g.A71023G and g.T72440C; g.T75757C and c.C1179T.

Although the results in many cases were not statistically significant, we observed different patterns of genetic segregation of alleles in control and tumor-affected subjects, which may serve as a risk factor of cancer occurrence in the investigated group. Moreover, g.T75757C and c.C1179T polymorphisms may be recognized as markers of aging in dogs.

Keywords: protein kinase, gene polymorphism, adenocarcinoma, bitches

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Recent results involving cellular and/or molecular mechanisms of neoplastic disease include the discovery of new molecules and their biochemical pathways in humans and laboratory mice (2, 3, 20, 31). In most cases the research on cancer development included analysis of defective genes, oncogenes and tumor suppressor genes that are associated with the disruption of expression of cell division proteins (1, 14, 26, 32). Moreover, these molecules (genes and/or proteins) are essential factors involved in neoplastic transformations of cells, malignant progression or lethal outcome of metastatic disease. Although several of these mechanisms are well recognized in humans or murine cancer development models, little is nevertheless known about the mechanisms and molecules that contribute to canine tumors (19, 22, 23). In many cases this deficiency is related to the small groups of investigated individuals as well as poor clinical evaluation of cancer or accompanying disease records. It was demonstrated that genes involved in tumor development or progression might be addressed separately, including (1) mediators of apoptosis and DNA repair, (2) oncogenes and tumor suppressors, (3) adhesion molecules, (4) mediators of angiogenesis, and (5) markers of circulating tumor cells (5, 6, 9-12, 21, 24, 30). Several of these mechanisms are regulated by expression of protein kinases that up-regulate their expression through the phosphorylation of target proteins. It may be assumed that protein kinases are involved in many mechanisms crucial for proper cell life or survival. Therefore, improper expression and/or genes sequence changes in protein kinases may be a key reason for induction of carcinogenesis.

In this study the gene polymorphisms of mitogen-activated protein kinase (*MAP2K1*) were analyzed. The MAP2K1, described also as extracellular signal-regulated kinase (ERKs), belongs to the family of protein kinases that are immediately phosphorylated on threonine and tyrosine residue and are highly conserved in evolution (8). MAP2K1 is also called a "checkpoint" or "integration point," because it is activated by multiple biochemical extracellular signals that promote the expression of target genes and proteins involved in cell growth and developmental progression.

Materials and methods

Subjects and samples collection. Blood samples were obtained from 22 female dogs before ovariohysterectomy diagnosed with mammary tumors during surgery in the Small Animal Clinic, University of Life Sciences, Poznan, Poland. Blood was collected during standard surgery procedures. 36 bitches served as controls. They were divided into 3 subgroups of differing age according to the classification by Jugdutt et al (15): geriatric (> 5 to 10-years-old; n = 15), mature adult (> 2 to 5-years-old; n = 10) and young (from 1 to 2-years-old; n = 11) dogs. Blood was collected from the jugular vein into vials with EDTA and frozen in –80°C until further analyses.

Tab. 1. PCR primer pairs and cycle conditions for the amplification of exons of *MAP2K1* gene

Exon	Sequences	Annealing temp./ elongation
exon 2	F 5'-CCCTGCATCCATTCTCCTAT-3'	58°C/30 sec
	R 5'-AATCCCATTCAGCCCTTTTT-3'	
exon 3	F 5'-CCTACCTTTAAGAGCGTAAGCA-3'	59°C/30 sec
	R 5'-GAGGCCCAAGACAGTGATGT-3'	
exon 4	F 5'-AAAGGGTTCCCTTCCAGAGA-3'	58°C/30 sec
	R 5'-CAAACTTTCCCTTGATTGACTT-3'	
exon 5	F 5'-GGGAAAAAGGCAAATTTGTGA-3'	58°C/30 sec
	R 5'-TCAATGGTTTTTACACCAAAACA-3'	
exon 6	F 5'-GTGGGATGGCACTACATCCT-3'	58°C/30 sec
	R 5'-TCCTCCCCCATTTCTTTTTC3'	
exon 7	F 5'-CTCAAAGGGGCAGGGAGT-3'	59°C/30 sec
	R 5'-CAATCCGTCTTGGGAGAATG-3'	
exon 8	F 5'-CATCCACCACCCTATTCTGG-3'	60°C/30 sec
	R 5'-GAATCCACAGGAACCCAAGA-3'	
exon 9, exon 10	F 5'-GGAGAAGGGCAGCAGTGTC-3'	60°C/60 sec
	R 5'-TTCCCTCAGCCTGTCCTTTA-3'	
exon 11	F 5'-TACAGCTACCCCAGGACAGG-3'	58°C/30 sec
	R 5'-TTGGCACATGCTGTGTTCTT-3'	

Molecular analyses. Genomic DNA was extracted from whole peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's instructions. DNA was suspended in 100 µl of Qiagen elution buffer and stored at –20°C. Ten exons of the *MAP2K1* gene (exon 2 – exon 11) were amplified in polymerase chain reaction (PCR), including up to 70-bp flanking regions of every exon. The sequences of primers used with thermal and time conditions are listed in Table 1. The reactions were carried out in a total volume of 12.5 µl containing: 10 × Taq DNA Polymerase buffer with $MgCl_2$, 5 × GC-rich solution, 0.24 mM dNTPs, 0.5 µM of the primers, 1 unit of Taq Polymerase (Roche) and 40-60 ng of genomic DNA. The PCR cycle conditions were as follows: an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at temperatures shown in Table 1 for 1 min and elongation at 72°C for 30/60 sec, with a final extension at 72°C for 7 min. PCR products were purified using membrane plates (Millipore) and used as templates in PCR-sequencing reamplification. The latter reaction was performed using Veriti 96 well Thermal Cycler and BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and one of the specific primers (a forward or reverse one). Reamplification products were purified by EDTA and ethanol precipitation and separated by electrophoresis using ABI 3130 sequencer (Applied Biosystems).

Statistical analyses. Genotypes obtained in the study were aligned with the reference sequences from Ensembl database. Single nucleotide variations were assessed and calculation of the chi-square test for deviation from Hardy-Weinberg equilibrium (HWE) was performed. Genotype and allele frequencies were evaluated and compared between study and control groups using Fisher's exact test. Odds ratio values (OR) were also evaluated and $p < 0.05$ was considered to indicate statistically significant differences. Additionally, we used Haploview 3.2 software to obtain *MAP2K2* gene structure. Linkage disequilibrium values (LD) were calculated as R^2 value and the Gabriel et al algorithm was used.

Results and discussion

We performed Sanger sequencing of 10 exons (exon 2 to exon 11) with approximately 70-bp non-coding splicing regions of *MAP2K2* gene in 58 subjects, in total. Upon alignment of obtained and reference sequences 15 single nucleotide variations were identified, of which 3 were situated in exons (exon 2, 7 and 11) and the remaining 12 were localized in splice regions of introns. The numbering of nucleotides was carried out with reference to the first nt in the first coding triplet of AUG in exon2. All single nucleotide variations identified in the study are listed in Table 2.

Tab. 2. Single nucleotide variations identified in *MAP2K1* gene in studied subjects

	Name of variation	Localization	nt change	aa change
1	c.G267T	exon 2	G > T	syn: P89P (CCG > CCT)
2	g.G48375T	intron 3	G > T	–
3	g.A48580G	intron 4	A > G	–
4	g.T68788C	intron 6	T > C	–
5	c.T795C	exon 7	T > C	syn: P265P (CCT > CCC)
6	g.G70977C	intron 7	G > C	–
7	g.G70978A	intron 7	G > A	–
8	g.A71023G	intron 7	A > G	–
9	g.T72440C	intron 7	T > C	–
10	g.T72562C	intron 8	T > C	–
11	g.G75654A	intron 9	G > A	–
12	g.C75727A	intron 10	C > A	–
13	g.T75728C	intron 10	T > C	–
14	g.T75757C	intron 10	T > C	–
15	c.C1179T	exon 11	C > T	syn: V393V (GTC > GTT)

All of the nucleotide changes were substitutions and were biallelic. All 3 substitutions in exon 2, 7 and 11 were synonymous and did not change the amino acid sequence; however, they could influence the folding of the mRNA and its biological interactions with other molecules, e.g. splicing factors.

We determined frequencies of alleles and genotypes for all 15 variants. Distribution of all *MAP2K1* genotypes was consistent with HWE. Frequencies and OR values for chosen variations are shown in Table 3. There were differences in genotype and allele frequencies between tumor and control groups for heterozygote and for an alternative allele for 3 polymorphisms (c.G267T, g.T72562C, g.T75728C). However, they were not statistically significant or were on the boundary of statistical significance, while the frequencies of allele and genotypes in control subgroups did not unambiguously indicate risk or protective variants.

Differences in allele and genotype frequencies between control subgroups were also inconclusive. We observed much higher prevalence of heterozygotes and alternative alleles in extreme subgroups, e.g. in the young and geriatric subgroups in comparison with the adult subgroup in 4 polymorphisms: g.G48375T, g.A48580G, c.T795C, g.T72562C. The odds ratio values were quite high (see Table 3) and the result for all of the variations were statistically significant or were on the boundary of significance. Moreover, the first two variations (g.G48375T, g.A48580G) were in 100% linkage and constituted a haplotype block (Figure 1). The prevalence of genotypes and alternative alleles in control and tumor patients for 3 of those 4 polymorphisms (g.G48375T, g.A48580G, c.T795C) did not differ. Interestingly, for the variation g.T72562C, the prevalence of the alter-

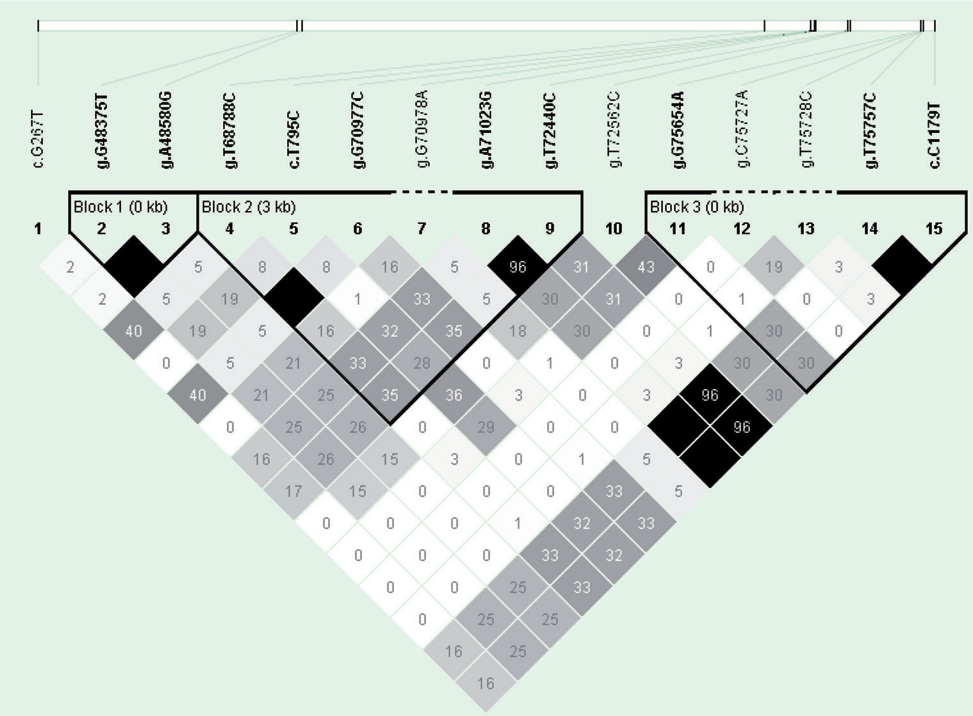

Fig. 1. Structure of *MAP2K1* gene shown as case-control association

Fig. 1 shows MAP2K1 gene structure displayed as case-association test. The relationship between every two single nucleotide variations are shown as linkage disequilibrium (LD), calculated as R2 value. Numbers in squares represent percentage of subjects in the study that were in linkage in reference to two variations crossed in each square. MAP2K1 gene structure revealed three haplotype blocks

Tab. 3. Frequencies and odds ratio values for chosen single nucleotide variations in *MAP2K1* gene (Y = young; A = adult; G = geriatric)

Variation		Tumor patients	Controls		p value	OR (95% CI)
g.G48375T	Genotype frequency	(n = 22)	(n = 36)			
	GG	16 (0.73)	22 (0.61)			
	GT	6 (0.27)	14 (0.39)	C vs. T	0.3687	1.7 (0.5-5.4)
			subgroup Y (n = 11)			
			GG 7 (0.64)			
			GT 4 (0.36)	G vs. Y	0.2386	2.6 (0.5-13.1)
			subgroup A (n = 10)			
			GG 9 (0.9)			
			GT 1 (0.1)	G vs. A	0.0272*	13.5 (1.3-135.9)
			subgroup G (n = 15)			
			GG 6 (0.4)			
			GT 9 (0.6)			
	Allele frequency					
	G	38 (0.86)	58 (0.8)			
	T	6 (0.14)	14 (0.2)	C vs. T	0.4238	1.5 (0.5-4.3)
			subgroup Y			
			G 18 (0.82)			
			T 4 (0.18)	Y vs. A	0.2165	4.2 (0.4-41.5)
			subgroup A			
			G 19 (0.95)			
			T 1 (0.05)			
			subgroup G			
			G 21 (0.7)			
			T 9 (0.3)	G vs. A	0.0567	8.1 (0.9-70.4)
c.T795C	Genotype frequency	(n = 22)	(n = 36)			
	TT	12 (0.55)	21 (0.58)			
	CT	9 (0.41)	13 (0.36)	T vs. C	0.715	1.23 (0.4-3.64)
	CC	1 (0.04)	2 (0.06)			
			subgroup Y (n = 11)			
			TT 6 (0.5)			
			CT5 (0.45)	Y vs. A	0.0974	7.5 (0.7-81.3)
			subgroup A (n = 10)			
			TT 9 (0.9)			
			CT 1 (0.1)			
			subgroup G (n = 15)			
			TT 6 (0.4)			
			CT 7 (0.47)	G vs. A	0.0789	7.8 (0.8-78.7)
			CC 2 (0.13)			
	Allele frequency					
	T	33 (0.75)	55 (0.76)			
	C	11 (0.25)	17 (0.24)	T vs. C	0.8653	1.1 (0.5-2.6)
			subgroup Y			
			T 17 (0.77)			
			C 5 (0.23)	Y vs. A	0.133	5.6 (0.6-52.7)
			subgroup A			
			T 19 (0.95)			
			C 1 (0.05)			
			subgroup G			
			T 19 (0.63)			
			C 11 (0.37)	G vs. A	0.0283*	11 (1.3-93.8)

Tab. 3. Continuation

Variation		Tumor patients	Controls		p value	OR (95% CI)
g.T72562C	Genotype frequency	(n = 22)	(n = 36)			
	TT	17 (0.77)	20 (0.56)			
	CT	5 (0.23)	14 (0.39)	C vs. T	0.208	2.2 (0.7-7.2)
	CC	0	2 (0.05)	C vs. T	0.4523	3.3 (0.2-71.1)
			subgroup Y (n = 11)			
			TT 5 (0.45)			
			CT 6 (0.55)	Y vs. A	0.0503	10.8 (1-117)
			subgroup A (n = 10)			
			TT 9 (0.9)			
			CT 1 (0.1)			
			subgroup G (n = 15)			
			TT 6 (0.4)			
			CT 7 (0.47)	G vs. A	0.0789	7.9 (0.7-7.2)
			CC 2 (0.13)			
	Allele frequency					
	T	39 (0.89)	54 (0.75)			
	C	5 (0.11)	18 (0.25)	C vs. T	0.0809	2.6 (0.9-7.6)
			subgroup Y			
			T 16 (0.73)			
			C 6 (0.23)	Y vs. A	0.0828	7.1 (0.8-65.6)
			subgroup A			
			T 19 (0.95)			
			C 1 (0.05)			
			subgroup G			
			T 19 (0.63)			
			C 11 (0.37)	G vs. A	0.0283*	11 (1.3-93.8)
g.T75757C	Genotype frequency	(n = 22)	(n = 36)			
	TT	8 (0.36)	9 (0.25)			
	CT	11 (0.5)	19 (0.53)			
	CC	3 (0.14)	8 (0.22)	C vs. T	0.4225	1.8 (0.4-7.7)
			subgroup Y (n = 11)			
			TT 4 (0.36)			
			CT 6 (0.55)			
			CC 1 (0.09)			
			subgroup A (n = 10)			
			TT 4 (0.4)			
			CT 4 (0.4)			
			CC 2 (0.2)	A vs. Y	0.4854	2.5 (0.2-32.8)
			subgroup G (n = 15)			
			TT 1 (0.07)			
			CT 9 (0.6)			
			CC 5 (0.33)	G vs. Y	0.1738	5 (0.5-50.8)
	Allele frequency					
	T	27 (0.61)	37 (0.51)			
	C	17 (0.39)	35 (0.49)	C vs. T	0.2956	1.5 (0.7-3.2)
			subgroup Y			
			T 14 (0.64)			
			C 8 (0.36)	G vs. Y	0.0578	3 (0.9-9.5)
			subgroup A			
			T 12 (0.6)			
			C 8 (0.4)	G vs. A	0.1085	2.6 (0.8-8.3)
			subgroup G			
			T 11 (0.37)			
			C 19 (0.63)			

Tab. 3. Continuation

Variation		Tumor patients	Controls		p value	OR (95% CI)
c.C1179T	Genotype frequency	(n = 22)	(n = 36)			
	CC	8 (0.36)	9 (0.25)			
	CT	11 (0.5)	19 (0.53)			
	TT	3 (0.14)	8 (0.22)	C vs. T	0.4225	1.8 (0.4-7.7)
			subgroup Y (n = 11)			
			CC 4 (0.36)			
			CT 6 (0.55)			
			TT 1 (0.09)	G vs. Y	0.1738	5 (0.5-50.8)
			subgroup A (n = 10)			
			CC 4 (0.4)			
			CT 4 (0.4)			
			TT 2 (0.2)	A vs. Y	0.4854	2.5 (0.2-32.8)
			subgroup G (n = 15)			
			CC 1 (0.07)			
			CT 9 (0.6)			
			TT 5 (0.33)	G vs. A	0.4711	2 (0.3-13.2)
	Allele frequency					
	C	27 (0.61)	37 (0.51)			
	T	17 (0.39)	35 (0.49)			
			subgroup Y			
			C 14 (0.64)			
			T 8 (0.36)	G vs. Y	0.0578	3 (0.9-9.5)
			subgroup A			
			C 12 (0.6)			
			T 8 (0.4)	G vs. A	0.1085	2.6 (0.8-8.3)
			subgroup G			
			C 11 (0.37)			
			T 19 (0.63)			

native homozygote CC, heterozygote CT and for the alternative allele C was over 2-fold higher in the controls than in the tumor group, and the latter odds ratio value was on the boundary of statistical significance.

We also observed higher prevalence of the alternative alleles and/or alternative homozygotes solely in the most advanced age subgroup in comparison with the other two subgroups for 2 pair of variations: g.A71023G and g.T72440C; g.T75757C and c.C1179T. A similar trend was observed in genotype distribution and the incidence of an alternative homozygote was 5-fold higher in the geriatric vs. young subgroup and 2-fold higher in the geriatric vs. adult subgroup. Some of the results were on the boundary of statistical significance, which in a larger population sample might indicate a reliable trend in allele distribution. Moreover, both pairs of polymorphisms were in 100% linkage. The distribution of alleles and genotypes for those two pairs of polymorphisms in control and tumor patient were comparable and did not differ significantly.

MAP2K1 gene structure of the case-control association study revealed 3 haplotype blocks, according to the Gabriel et al. algorithm (Figure 1). Haplotype block 1 involved only two polymorphisms (g.G48375T in intron 3 and g.A48580G in intron 4) with linkage disequilibrium in 100% of individuals studied. Similarly, there was 100% linkage between variations g.T75757C in intron 10 and c.C1179T in exon 11. In haplotype block 2 we observed 100% linkage between polymorphisms g.T68788C and g.G70977C, and 96% linkage between the other two: g.A71023G and g.T72440C. There was also a significantly pronounced linkage between 4 other pairs of variations shown in the case-control study: between g.A71023G and g.T75757C and between g.A71023G and c.C1179T in 100% of individuals, and between g.T72440C and g.T75757C and between g.T72440C and c.C1179T in 96% of individuals. Interestingly, there was 52% and 50% linkage between polymorphisms c.T795C and g.T72562C, and c.T795C and g.G75654A, respectively, seen in *MAP2K1* gene structure in the control group.

Many definitions of carcinogenesis exist but the main one includes the process of uncontrolled cell division, which often leads to cancer growth and development. In many cases uncontrolled cell division is a result of improper expression of genes and proteins that are responsible for control of cell division, since they are also called a "checkpoint of cell division" (4, 18, 25, 28, 29). Moreover, in many cases down-, and/or up-regulation of gene expression is associated with changes in gene sequence, such as gene mutation and/or polymorphisms. In this study, we analyzed the occurrence of mutation and *MAP2K1* polymorphism in the selected population of bitches with diagnosed adenocarcinoma. Furthermore, the frequencies of *MAP2K1* gene polymorphisms were also analyzed in the aged group of investigated subjects. Since MAP2K1 belongs to the mitogen-activated kinase family that is responsible for cell division cycle control, we suggested that changes in the gene structure such as polymorphism may lead to uncontrolled cell divisions and carcinogenesis induction and/or growth and development of cancer in aged bitches. Although in many cases we did not find any statistically significant association between occurrence of adenocarcinoma and aging in bitches, we could postulate that some of the polymorphisms (3 substitutions in exon 2, 7 and 11) may influence slicing and posttranscriptional mRNA processing that leads to improper protein synthesis or folding. Moreover, some of the polymorphisms may also play a protective or a risk role during aging in dogs. In addition, two polymorphisms, g.T75757C and c.C1179T, displayed a 2.6-fold higher prevalence of an alternative allele in the geriatric subgroup than in the adult subgroup and a 3-fold higher prevalence in the geriatric vs. young subgroup. It may indicate that these changes may be recognized as risk factors in aging and/or be markers in aged bitches. However, we also detected such polymorphisms that cannot serve as markers of aging because they displayed similar allelic frequency and association in young and geriatric subgroups. In addition, g.T72562C polymorphism did not indicate a putative protect variant, as the differences in frequencies between control subgroups showed very high and significant incidence of the allele C in both young and geriatric subgroups.

We also found that several changes out of all observed in *MAP2K1* showed a different pattern of segregation between the bitches with adenocarcinoma and in control animals. In addition, polymorphic variants c.T795C and g.T72562C, c.T795C and g.G75654A, although not statistically significant themselves, were 3- and 10-fold higher, respectively, than the linkage between those two pairs of variations seen in the gene structure of tumor patients. Therefore, it may suggest a slightly different pattern of genetic segregation of alleles in control and tumor-affected subjects.

The available data indicate that the incidence and occurrence of *MAP2K1* or *MEK1* kinase gene polymorphisms can be noted in many types of human cancers, including leukemia, liver and gastric cancer (13, 16, 17, 33). In the recent few years a multiple next generation sequencing panel has often been used for detection of new mutations in genes associated with the occurrence of cancers. Scarpa A., et al. (27) performed molecular analysis of lung adenocarcinoma on cytological samples and observed 504 mutational hotspots in the structure of 22 lung cancer related marker genes. In 24/36 cases (67%) at least one mutated gene was found, including growth factor receptors, secondary transduction proteins and *MAP2K1* as the cell division cycle "checkpoint" protein. They concluded that those mutations may be recognized as markers of occurrence of lung adenocarcinoma as well as next generation sequencing methods may be used as a reference procedure for the introduction of personalized therapy in adenocarcinoma patients. In the other study obtained by Choi et al. (6), a retroviral complementary DNA (cDNA) expression library was constructed from a cell line (OCUM-1) of scirrhous gastric cancer. They used mouse fibroblast 3T3 cell line to generate a transformed cDNA encoding for *MAP2K1*, characterized by glutamine-to-proline substitution at amino acid position 56. They observed that treatment of OCUM-1 cell line with MAP2K1 specific inhibitor induced cell death. However, this effect was not observed in the scirrhous gastric cancer that did not carry *MAP2K1* mutations. It was suggested that the *MAP2K1* mutation significantly influenced induction of carcinogenesis. Moreover, after using a next-generation sequencing analysis, they detected one known (D67N) and four novel (R47Q, R49L, I204T and P306H) mutations within *MAP2K1*, which also played a crucial role during induction of carcinogenesis.

In conclusion, since MAP2K1 is a key protein that regulates the cell division cycle and proliferation, mutation and/or polymorphisms in the gene coding sequence may be, as it was described above, a major reason of uncontrolled cell division. Moreover, it may lead to induction of carcinogenesis as well as growth, development and metastases of cancer in both human and other mammalian species, including dogs.

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