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ORIGINAL ARTICLE

Frameshift Mutations of *AKAP9* Gene in Gastric and Colorectal Cancers with High Microsatellite Instability

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Abstract A-kinase-anchoring protein 9 (AKAP9) coordinates the cellular location and function of protein kinase A. AKAP9 plays an important role in centrosome duplication, cell cycle progression and maintenance of cell membrane integrity, alterations of which contribute to tumorigenesis. Somatic mutations of *AKAP9* gene have been detected in many cancers including gastric (GC) and colorectal cancers (CRC), but the mutation status with respect to microsatellite instability (MSI) has not been reported. In a public database, we found that *AKAP9* gene had mononucleotide repeats in the coding sequences that might be mutation targets in the cancers with MSI. We analyzed the mutations in 79 GCs and 124 CRCs including high MSI (MSI-H) and microsatellite stable/low MSI (MSS/MSI-L) cases by single-strand conformation polymorphism analysis and DNA sequencing. Overall, we found *AKAP9* frameshift mutations in 4 (11.7 %) GCs and 20 (17.7 %) CRCs with MSI-H (24/113), but not in MSS/MSI-L cancers (0/90) ($p < 0.001$). In addition, we analyzed intratumoral heterogeneity (ITH) of *AKAP9* frameshift mutations in 16 CRCs and found that five CRCs (31.3 %) harbored regional ITH of the *AKAP9* frameshift mutations. Our data indicate that *AKAP9* gene harbors not only somatic frameshift mutations but also mutational ITH, which together may be features of GC and CRC with MSI-H. Our results also suggest that regional mutation analysis is needed for a better evaluation of mutation status in these tumors to overcome ITH.

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Introduction

Intracellular organization of protein kinase A (PKA) is regulated through association with A-kinase-anchoring proteins (AKAPs) that have the common functions of binding to the regulatory subunit of PKA and confining the PKA to discrete cellular locations [1, 2]. AKAP9, also known as AKAP450/AKAP350/CG-NAP localizes at centrosome and Golgi apparatus where it assembles proteins such as PKA, Cdk2, Ran small GTPase and the γ-TURC associated proteins GCP2/3 [1, 2]. AKAP9 contributes to centrosome duplication and G_1/S cell cycle progression that are important in tumorigenesis [3–5]. Also, AKAP-mediated organization of kinases is important for the signal transmission to cytoskeleton [6–9]. For example, AKAP9 binds with E-cadherin and maintains membrane integrity and protects epithelial barriers [9]. Loss of E-cadherin by smoking is accompanied by reduced AKAP9 protein expression at the cell membrane [9]. Sequence analysis has identified multiple alternatively spliced *AKAP9* isoforms, which are not only expressed in centrosome and Golgi apparatus, but also in other intracellular structures [10]. In cancers, *AKAP9-BRAF* fusion is common in radiation-related thyroid cancers [11]. In addition, *AKAP9* somatic mutations are frequent in many cancers including oral squamous cell carcinoma [12]. Together, these data suggest that physiological function of AKAP9 is identified in various tissues and alterations of *AKAP9* are possibly involved in tumorigenesis. However, the functional consequences of the *AKAP9* mutations have not been explored.

In a public genome database (http://genome.cse.ucsc.edu/), we found that *AKAP9* gene has mononucleotide repeats in

their coding sequences that could be targets for frameshift mutation in cancers with microsatellite instability (MSI). Frameshift mutations of genes with mononucleotide repeats are features of gastric (GC) and colorectal cancers (CRC) with microsatellite instability (MSI) [13], but *AKAP9* mutational status in GC and CRC with MSI has not been explored. Cancer development begins with clonal expansion of a single cell and the resulting cell population usually becomes heterogeneous after branching sub-clonal expansion, which leads to intratumoral heterogeneity (ITH) [14]. In this study, we analyzed somatic frameshift mutation of *AKAP9* gene and its mutational ITH in GC and CRC.

Materials and Methods

Tissue Samples and Microdissection

For mutation analysis, 79 sporadic GCs and 124 sporadic CRCs were used in this study. Of them, 54 CRCs were frozen tissues and the other 149 tissues were methacarn-fixed tissues. The GCs consisted of 34 GCs with high MSI (MSI-H), 45 GCs with microsatellite stable/low MSI (MSS/MSI-L), 79 CRCs with MSI-H and 45 CRCs with MSS/MSI-L. The MSI evaluation system used five mononucleotide repeats (BAT25, BAT26, NR-21, NR-24 and MONO-27), tumoral MSI status of which was characterized as: MSI-H, if two or more of these markers show instability, MSI-L, if one of the markers shows instability and MSS, if none of the markers shows instability [15]. For 54 CRCs, we collected four to seven different tumor areas and one normal mucosal area from each fresh CRC specimen to analyze the mutational ITH. The tumor areas were 0.027–1 cm^3 and at least 1.0 cm apart from each other. To confirm that these multi-regional biopsies were all areas of carcinoma (as opposed to areas of normal or dysplasia), they were frozen, stained with hematoxylin and eosin and examined under light microscope. The tumor cell purities of the ITH tissues were at least 70 %. Sixteen of the 54 CRCs with ITH collection were identified as MSI-H. These four to seven different tumor areas in the 16 CRCs were used for detecting regional ITH of *AKAP9* gene. Pathologic features of the cancers are summarized in Table 1. The histologic features of CRC with MSI-H, including mucinous histology, tumor infiltrating lymphocytes, medullary pattern, and Crohn's like inflammation, were evaluated in all blocks of all cases by a pathologist. Malignant cells and normal cells were selectively procured from hematoxylin and eosin-stained slides using a 30G1/2 hypodermic needle by microdissection as described previously [16, 17]. DNA extraction was performed by a modified single-step DNA extraction method by proteinase K treatment. Approval of this study was obtained from the Catholic University of Korea, College of Medicine's institutional review board for this study.

Single Strand Conformation Polymorphism (SSCP) Analysis

We analyzed mononucleotide repeats of *AKAP9* in their coding sequences (an A8 in exon 2, an A7 in exon 31 and a G7 in exon 46). Genomic DNA from the microdissected cells was isolated, and was amplified by polymerase chain reaction (PCR) with specific primer pairs. Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. After SSCP, mobility shifts on the SSCP gels (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) were determined by visual inspection. Direct DNA sequencing reactions in both forward and reverse sequences were performed in the cancers with the mobility shifts in the SSCP using a capillary automatic sequencer (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA). When mutations in the genes were suspected by SSCP, analysis of an independently

Table 1 Summary of pathologic features of the cancers

No. of gastric carcinomas				No. of colorectal carcinomas			
		MSI-H (n = 34)	MSS/MSI-L (n = 45)			MSI-H (n = 79)	MSS/MSI-L (n = 45)
TNM	I	13	15	TNM	I	15	6
	II	13	18		II	29	20
	III	7	11		III	32	16
	IV	1	1		IV	3	3
Lauren's subtype	Diffuse	20	25	Location (colon)	Cecum	16	0
	Intestinal	14	20		Ascending	46	3
					Transverse	14	2
EGC Vs. AGC	EGC	3	4		Descending & sigmoid	3	17
	AGC	31	41		Rectum	0	23

EGC early gastric cancer, *AGC* advanced gastric cancer, *TNM* tumor, lymph node, metastasis

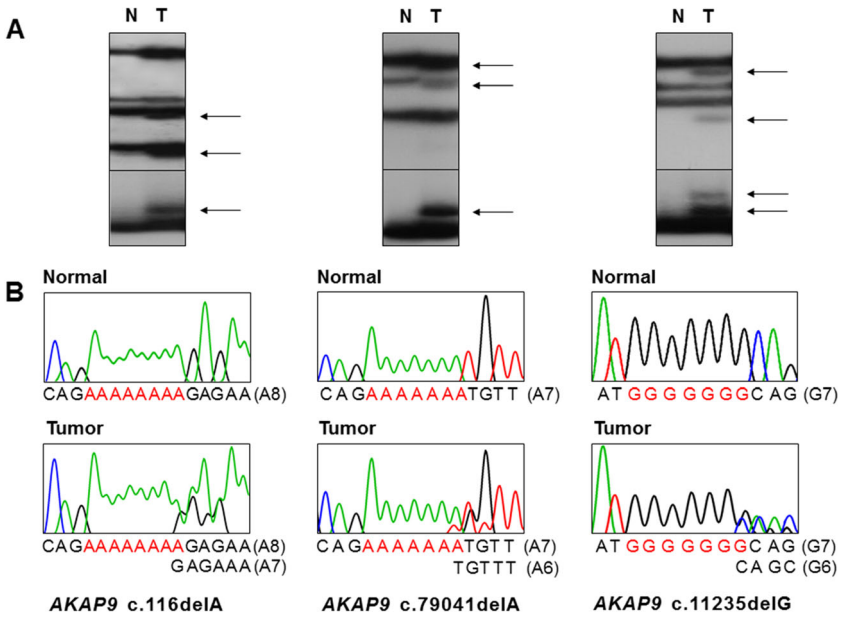

Fig. 1 Representative SSCP and DNA sequencings of repeats in *AKAP9* in colon and gastric carcinomas. SSCP (**a**) and DNA sequencing analyses (**b**) of *AKAP9* from tumor (Lane T) and normal tissues (Lane N). A. SSCPs of the PCR products from A8 repeat (left), A7 repeat (middle) and G7 repeat (right) show aberrant bands (arrows in lane T) as compared to SSCP from normal tissues (N). B. Direct DNA sequencing analyses (B) show heterozygous A deletion in the A8 (left), A deletion in the A7 (middle) and G deletion in the G7 (right) in tumor tissues as compared to normal tissues

isolated DNA from another tissue section of the same patients was performed to exclude potential artifacts originated from PCR. Other procedures for PCR-SSCP were described in our previous reports [15–17].

Results and Discussion

Genomic DNAs isolated from normal and tumor tissues of the 79 GC and 124 CRC were analyzed for detection of mutation in the nucleotide repeats (an A8 in exon 2, an A7 in exon 31 and a G7 in exon 46) of *AKAP9* by PCR-SSCP analysis. On the SSCP, we found aberrantly migrating bands in 19 cases of the A8, one case of the A7 and four cases of the G7 (Fig. 1 and Table 2). DNA from the normal tissues from corresponding patients showed no evidence of aberrant migration on SSCP, indicating the mutations had risen somatically (Fig. 1a). Direct DNA sequencing of the cancer tissues with the aberrant bands on SSCP confirmed that they represented somatic mutations of *AKAP9* gene (Fig. 1b). All of the mutations detected were interpreted as heterozygous according to the SSCP and direct sequencing analyses (Fig. 1a and b). All of the mutations were deletion or duplication mutations in the repeats that would cause premature stops, which would lead to termination of amino acid translation (Table 2).

The mutations were detected in cancers with MSI-H, but not in those with MSS/MSI-L (Table 2). There was a statistical difference in the frameshift mutation frequencies between the cancers with MSI-H (24/113) and MSS/MSI-L (0/90) (Fisher's exact test, $p < 0.001$). In terms of tissue origins, there was no statistical difference in mutation frequencies between GCs and CRCs (Fisher's exact test, $p = 0.056$). There was no significant association of the mutations with the clinicopathologic data of the patients (age, sex, histologic grade and stage). In the cancers with MSI-H, there was no correlation between histological features of the tumors (histologic grade, subtypes, mucinous histology, medullary pattern and tumor-infiltrating lymphocytes) and presence of the mutations.

From 96 regional fragments of 16 CRCs (4–7 fragments per case) with MSI-H were collected and analyzed with respect to their regional status of the frameshift mutations of *AKAP9*. Of the three repeats, the A8 repeat exhibited the deletion (A8 to A7) mutation in six of the 16 CRCs. Of the six CRCs, five harbored ITH of the deletion mutation (#3, 39, 41,

Table 2 Summary of *AKAP9* mutations in gastric and colorectal cancers

Gene	Location	Wild type	Mutation	MSI status of the mutation cases (n)	Incidence in MSI-H cancers (%)	Nucleotide change (predicted amino acid change)
AKAP9	Exon 2	A8	A7	MSI-H (18)	Gastric: 4/34 (11.7) Colorectal: 14/79 (17.7)	c.116delA (p.Lys39ArgfsX17)
		A8	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.115_116delAA (p.Lys39GlufsX9)
	Exon 31	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.7904delA (p.Asn2635MetfsX3)
	Exon 37	G7	G8	MSI-H (3)	Colorectal: 3/79 (3.8)	c.11235dupG (p.Gln3746AlafsX52)
		G7	G6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.11235delG (p.Gln3746SerfsX7)

Table 3 Intratumoral heterogeneity of *AKAP9* mutations in colorectal cancers

Case	Regional biopsy sites							Mutation status	ITH status
	#1	#2	#3	#4	#5	#6	#7		
CRC3	Wild type	Wild type	Wild type	c.116delA	Wild type	Wild type	n.d.	Mutation	ITH
CRC15	c.116delA	c.116delA	c.116delA	c.116delA	c.116delA	c.116delA	c.116delA	Mutation	Non-ITH
CRC26	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC27	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC34	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC35	Wild type	Wild type	n.d.	n.d.	n.d.	Wild type	Wild type	Wild type	-
CRC39	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	c.116delA	Mutation	ITH
CRC41	c.116delA	n.d.	c.116delA	c.116delA	n.d.	Wild type	c.116delA	Mutation	ITH
CRC43	Wild type	Wild type	Wild type	n.d.	n.d.	Wild type	n.d.	Wild type	-
CRC45	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC47	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC48	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC49	n.d.	c.116delA	c.116delA	c.116delA	Wild type	c.116delA	c.116delA	Mutation	ITH
CRC51	c.116delA	c.116delA	Wild type	c.116delA	c.116delA	c.116delA	c.116delA	Mutation	ITH
CRC53	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC55	Wild type	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type	Wild type	-

ITH: intratumoral heterogeneity

49 and 51) while one CRC (#15) exhibited the deletion in all regional biopsies. For example, a CRC case (#51) showed the deletion mutation in six (51–1, −2, −4, −5, −6 and −7) of seven regional biopsies (Table 3 and Fig. 2).

The Pan-Cancer analysis integrated overall genome sequencing data across cancer types and identified molecular commonalities [18–20]. Many mutations that occurred at low frequencies in a single cancer type were shared by sets of types and were considered significantly mutated genes [18–20]. *AKAP9* mutation has been observed in many cancers with low to moderate incidences (2.62 % in CRCs and 8.1 % in GCs) [20], but it became a significantly mutated gene by the Pan-Cancer analysis (search at www.intogen.org). In GCs and CRCs, one third of the non-silent mutations were frameshift mutations, while the other two thirds were missense mutations [20]. In the present study, we found frameshift mutations of

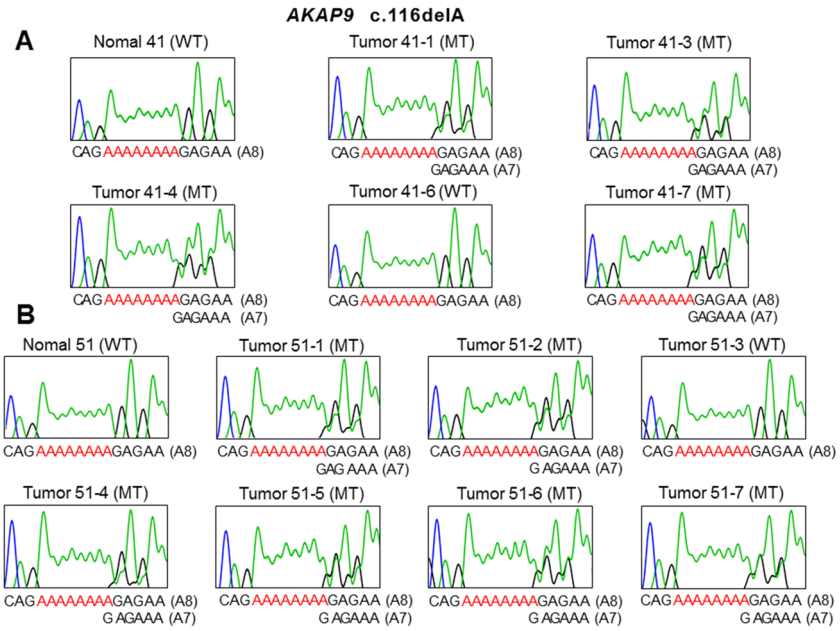

Fig. 2 Intratumoral heterogeneity of an *AKAP9* frameshift mutation in a colon cancer. **a** Direct DNA sequencings show *AKAP9* c.116delA mutation (MT) in four regional biopsies (41–1, −3, −4 and −7) and wild-type (WT) in the other one regional biopsy (41–6). **b** Direct DNA sequencings show *AKAP9* c.116delA mutation (MT) in six regional biopsies (51–1,-2, −4, −5, −6 and −7) and wild-type (WT) in the other one regional biopsy (51–3)

AKAP9 as well (Table 2), which would alter and delete amino acids after the frameshift mutations and hence resembled a typical loss-of-function mutation. Presence of the frameshift mutations in GCs and CRCs suggests that loss of AKAP9 functions might be related to the features of GC and CRC. Previous works identified that AKAP9 played a role in centrosome duplication and G_1/S cell cycle progression, suggesting its oncogenic activities [3–5]. On the other hand, AKAP9 cooperates with E-cadherin and protects epithelial barriers from carcinogens, suggesting its tumor suppressor activity [9]. However, because to our knowledge there is no definite experimental data on AKAP9 functions in tumor development, it is not possible to guess consequences of the frameshift mutations of *AKAP9*. Together, we suggest that AKAP9 may have two opposing tumor-related functions depending on the cellular context and that inactivating mutations of *AKAP* may possibly play a role in GC and CRC with MSI-H as a tumor suppressor. The information on *AKAP9* frameshift mutations in GC and CRC may provide basis for further efforts to elucidate its roles in pathological status. In the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic) that catalogues somatic mutations in cancers, we found *AKAP9* somatic mutations in many types of cancers, including colon, stomach, lung, skin and head/neck cancers [12, 18–20], suggesting that *AKAP9* alterations might be causally related to cancer pathogenesis.

Under proper conditions, the SSCP is able to detect over 90 % of mutations occurring within any sequence, and the sensitivity is believed to be high if the PCR fragments are shorter than 200 bp [21]. As we analyzed the samples by SSCP with products shorter than 200 bp in this study, the missing of *AKAP9* mutations, if any, would be very rare in the current study. In addition, to confirm the mutations, we repeated the PCR-SSCP twice. In the second round of SSCP, we included the positive controls of the mutations that had been detected in the first round SSCP.

In the present study we found ITH of *AKAP9* mutation in 5 of 16 CRCs (31.3 %). Our results are in agreement with earlier reports showing that mutational ITH within coding genes was encountered in CRCs with MSI-H [22]. The generation of ITH may influence on clinical outcome of the cancer patients. For example, low frequent mutations with a potential to metastasize define clinical outcomes since the clones with ITH easily achieve clonal dominance during the progression and affect treatment efficacy [14]. Probably due to the small number of cases with the ITH ($n = 5$), we were not able to define clinical feature of the mutational ITH of *AKAP9* mutation and it is needed that a larger cohort should be analyze to define the clinical features of the ITH. In the context of pathology practice, our data also suggest that there could be under- or over-estimation of frameshift mutations in the repeat sequences in cancers with MSI-H. Therefore, when performing mutation analysis in cancers with MSI-H, multi-regional biopsies should be taken into account for a better evaluation of the mutation status.

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References

1. Colledge M, Scotte JD (1999) AKAPs: from structure to function. Trends Cell Biol 9:216–221
2. Rubin CS (1994) A kinase anchor proteins and the intracellular targeting of signals carried by cyclic AMP. Biochim Biophys Acta 1224:467–479
3. Schmidt PH, Dransfield DT, Claudio JO, Hawley RG, Trotter KW, Milgram SL, Goldenring JR (1999) AKAP350, a multiply spliced protein kinase a-anchoring protein associated with centrosomes. J Biol Chem 274(5):3055–3066
4. Terrin A, Monterisi S, Stangherlin A, Zoccarato A, Koschinski A, Surdo NC, Mongillo M, Sawa A, Jordanides NE, Mountford JC, Zaccolo M (2012) PKA and PDE4D3 anchoring to AKAP9 provides distinct regulation of cAMP signals at the centrosome. J Cell Biol 198:607–621
5. Mattaloni SM, Ferretti AC, Tonucci FM, Favre C, Goldenring JR, Larocca MC (2013) Centrosomal AKAP350 modulates the G1/S transition. Cell Logist 3:e26331
6. Diviani D, Scott JD (2001) AKAP signaling complexes at the cytoskeleton. J Cell Sci 114:1431–1437
7. Scotte JD (2003) A-kinase-anchoring proteins and cytoskeletal signalling events. Biochem Soc Trans 31(Pt 1):87–89
8. Gros R, Ding Q, Chorazyczewski J, Pickering JG, Limbird LE, Feldman RD (2006) Adenylyl cyclase isoform-selective regulation of vascular smooth muscle proliferation and cytoskeletal reorganization. Circ Res 99:845–852
9. Oldenburger A, Poppinga WJ, Kos F, de Bruin HG, Rijks WF, Heijink IH, Timens W, Meurs H, Maarsingh H, Schmidt M (2014) A-kinase anchoring proteins contribute to loss of E-cadherin and bronchial epithelial barrier by cigarette smoke. Am J Physiol Cell Physiol 306:C585–C597
10. Chen L, Marquardt ML, Tester DJ, Sampson KJ, Ackerman MJ, Kass RS (2007) Mutation of an a-kinase-anchoring protein causes long-QT syndrome. Proc Natl Acad Sci U S A 104:20990–20995
11. Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z, Nikiforova MN, Rabes HM, Fagin JA, Nikiforov YE (2005) Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J Clin Invest 115:94–101
12. Onken MD, Winkler AE, Kanchi KL, Chalivendra V, Law JH, Rickert CG, Kallogjeri D, Judd NP, Dunn GP, Piccirillo JF, Lewis JS Jr, Mardis ER, Uppaluri R (2014) A surprising cross-species conservation in the genomic landscape of mouse and human oral cancer identifies a transcriptional signature predicting metastatic disease. Clin Cancer Res 20:2873–2884
13. Imai K, Yamamoto H (2008) Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. Carcinogenesis 29:673–680
14. Marusyk A, Almendro V, Polyak K (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12:323–334
15. Murphy K, Zhang S, Geiger T, Hafez MJ, Bacher J, Berg KD, Eshleman JR (2006) Comparison of the microsatellite instability analysis system and the bethesda panel for the determination of microsatellite instability in colorectal cancers. J Mol Diagn 8:305–311

16. Oh HR, An CH, Yoo NJ, Lee SH (2015) Frameshift mutations of MUC15 gene in gastric and its regional heterogeneity in gastric and colorectal cancers. Pathol Oncol Res 21: 713–718
17. Choi MR, An CH, Yoo NJ, Lee SH (2015) Laminin gene LAMB4 is somatically mutated and expressionally altered in gastric and colorectal cancers. APMIS 123:65–71
18. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, Leiserson MD, Miller CA, Welch JS, Walter MJ, Wendl MC, Ley TJ, Wilson RK, Raphael BJ, Ding L (2013) Mutational landscape and significance across 12 major cancer types. Nature 502:333–339
19. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G (2014) Discovery and saturation analysis of cancer genes across 21 tumour types. Nature 505:495–501
20. Gonzalez-Perez A, Perez-Llamas C, Deu-Pons J, Tamborero D, Schroeder MP, Jene-Sanz A, Santos A, Lopez-Bigas N (2013) IntOGen-mutations identifies cancer drivers across tumor types. Nat Methods 10:1081–1082
21. Hayashi K (1992) PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl 1: 34–38
22. Calin GA, Gafà R, Tibiletti MG, Herlea V, Becheanu G, Cavazzini L, Barbanti-Brodano G, Nenci I, Negrini M, Lanza G (2000) Genetic progression in microsatellite instability high (MSI-H) colon cancers correlates with clinico-pathological parameters: a study of the TGRbetaRII, BAX, hMSH3, hMSH6, IGFIIR and BLM genes. Int J Cancer 89:230–235