a higher transcriptional activity.

Discussion

Approximately 160 million people (2.35% of the worldwide population) are estimated to have HCV infection [27]. Since HCV carriers have an increased risk to develop liver cirrhosis and subsequent HCC [28,29], the prediction of cancer risk is especially important for CHC patients. In our previous study, we have identified that SNP rs2596542 located in the upstream of *MICA* gene was significantly associated with the risk of HCC development among CHC patients as well as the serum level of sMICA [6]. In this study, we found that the genetic variant at SNP rs2596538 strongly affected the binding affinity of SP1. Over-expression of SP1 remarkably induced *MICA* expression in cells carrying the G allele that has a higher affinity to the SP1 binding. These findings are concordant with higher serum sMICA level among HCC patients with the G allele at SNP rs2596538. SP1 is a ubiquitously expressed transcription factor which binds to the GC-rich decanucleotide sequence (GC box) and activates the transcription of various viral and cellular genes [30,31]. Phosphorylation of SP1 was shown to be induced by HCV core protein and exhibited higher binding affinity to the promoter region of its downstream targets [32]. From our previous study, we showed a significant difference

of sMICA expression between non-HCV individuals and CHC patients. This indicated that sMICA expression was induced after HCV infection [6]. Hence, we here propose the following hypothesis. After HCV infection, the virus core protein enhances the SP1 phosphorylation in hepatocytes, and the phosphorylated SP1 binds to the DNA segment corresponding to the G allele of SNP rs2596538 and then induces *MICA* expression. The membrane-bound MICA (mMICA) serves as a ligand for NKG2D to activate the immune system and results in the elimination of viral-infected cells by NK cells and CD8+ T cells [8,9]. Eventually, HCV-infected individuals with higher MICA level may cause stronger immune response to the infected cells and hence result in a reduced risk for HCC progression. Moreover, the mMICA is then shed by metalloproteinases that are often over-expressed in cancer tissues and convert mMICA to sMICA. This resulted in a significantly increase of sMICA level in the serum of HCV infected patients.

In contrast to HCV-induced HCC, our group had previously identified that higher sMICA level was associated with poor prognosis in HBV-induced HCC patients [33]. Such an opposite effect of *MICA* would be attributable to the difference in downstream pathway between HBV and HCV. HBV virus encodes hepatitis B virus X protein (HBx) that is pathogenic and promotes tumor formation. It had been reported that HBx protein was associated with an elevated expression of MT1-MMP, MMP2, and MMP3 [34,35].

HBx was also shown to transactivate MMP9 through ERKs and PI-3K-AKT/PKB pathway and suppress TIMP1 and TIMP3 activities [36,37]. The activation of metalloproteinases would induce the shedding of mMICA into sMICA, which promotes the tumor formation through the inhibitory effect of sMICA on NK cells. This can explain why high sMICA expression is a marker of poor prognosis for HBV-induced HCC. On the other hand, HCV infection was not associated with metalloproteinases activation, although the expression of sMICA was shown to be proportional to mMICA level. Therefore individuals with high MICA expression are likely to activate natural killer cells and CD8+ T cells to eliminate virus infected cells.

SP1 was previously identified as a transcriptional regulator of both *MICA* and *MICB* [7,9,38]. A polymorphism in the *MICB* promoter region was found to be associated with *MICB* transcription level [7]. To our knowledge, this is the first report showing that *MICA* transcription is directly influenced by functional variant. Moreover, this functional SNP is significantly associated with HCV-induced HCC. Our findings provide an insight that *MICA* genetic variation is a promising prognostic biomarker for CHC patients.

Acknowledgements

This work was conducted as a part of the BioBank Japan Project that was supported by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government. This work was also supported by the grants from the Ministry of Education, Culture, Sports, Science and Technology, and Ministry of Health, Labour, and Welfare of the Japanese government.

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Figure legends

Fig. 1. SNP rs2596538 affects the binding affinity of nuclear proteins. (**A**) Real-time quantitative PCR (upper) and Western blotting (lower) of MICA before and after heat shock treatment in HLE cells. *B2M* and β-actin are served as internal and protein loading control. (**B**) EMSA using 31 bp labeled probes flanking each SNP located within the 4.8 kb region upstream of *MICA* transcription start site. A black arrow indicates the shifted band specific to G allele of SNP rs2596538. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled A or G allele of SNP rs2596538 at different concentrations are used as competitors. Pointed arrow indicates shifted band. $*P < 0.05$ by Student`s *t*-test.

Fig. 2. Binding of transcription factor SP1 to G allele of SNP rs2596538. (**A**) Multiple alignment of a GC box and DNA sequence of A or G probe of rs2596538 used in EMSA. (**B**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled consensus oligonucleotides of seven transcription factors are used as competitors. Pointed arrow indicates shifted band. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat shock treated HLE cells in the presence of anti-SP1 antibody or normal rabbit IgG. Asterisks

on the left side indicate the shifted (*) and super-shifted bands (**). Normal rabbit IgG serves as a negative control. (**D**) ChIP assay using HepG2 and HLE cell lines were ectopically expressed with SP1 protein. DNA-protein complex was immunoprecipitated with anti-SP1 antibody followed by PCR amplification using a primer pair flanking SNP rs2596538. DNAs precipitated without antibody are served as a negative control. PCR primers flanking the 3' UTR region of *MICA* are served as a negative control. (**E**) Genotype distribution at SNP rs2596538 in PCR fragment amplified from the input genomic DNA and DNA-protein complex immunopurified from HepG2 cells by using anit-SP1 antibody. *$P < 0.05$ by Student`s *t*-test.

Fig. 3. Transcriptional regulation of MICA by SP1 through genomic region including SNP rs2596538. (**A**) Reporter assay using constructs including 3 copies of 31 bp DNA fragment flanking SNP rs2596538. Reporter constructs are transfected into HLE cells with pRL-TK and pCAGGS or pCAGGS-SP1-HA vector. The value of relative luciferase activity was calculated as the firefly luciferase intensity divided by the renilla luciferase intensity. The data represent the mean ± SD value of 4 independent studies. (*$P < 0.05$, Student`s *t*-test) (**B**) MICA expression in HLE cells after

transfection with pCAGGS or pCAGGS-SP1 vector. β-actin is served as a protein loading control.

Fig. 4. Association between the soluble MICA levels and rs2596538 genotype. The samples were classified into 3 groups according to rs2596538 genotype. The sMICA levels measured by ELISA are indicated in y-axis. The numbers of samples and the proportion of sMICA positive subjects from each group are shown in x-axis. The percentage of the positive sMICA expression in each group are AA = 10%, AG = 39%, and GG = 42%. Statistical significance was determined by Kruskal-Wallis test.

Supplementary Fig. 1. Pairwise LD map between marker SNP and 11 candidates SNP. Black color boxes represent regions of high pairwise r^2 value. The LD was determined by direct DNA sequencing of *MICA* promoter region from 50 randomly selected HCV-HCC patients.

Table 1 Association of rs2596542 with the progression from CHC to LC and HCC

	Case MAF	Control MAF	*P**	OR	95% C.I.
LC vs CHC	0.3797	0.3442	0.04842	1.166	1.01-1.35
HCC vs LC	0.4012	0.3797	0.20296	1.094	0.95-1.26

MAF, minor allele frequency; OR, odds ratio for minor allele. C.I., confidence interval. SNP rs2596542 was analyzed in 1043 chronic hepatitis C (CHC), 586 liver cirrhosis without hepatocellular carcinoma (LC) and 1629 HCV-induce hepaticellular carcinoma patients (HCC). * calculated by Armitage trend test.

Table 2 Linkage disequilibrium between 11 candidate SNPs and SNP rs2596542

SNP ID	Relative position [a]	A1	A1 frequency	$D`$	r^2
rs2596542	-4815	A	0.36		
rs2428475	-4788	G	0.36	1	1
rs28366144	-4586	T	0.36	1	1
rs2428474	-4387	G	0.39	1	0.88
rs2251731	-4045	A	0.39	1	0.88
rs2844526	-3703	C	0.38	1	0.918
rs2596541	-3572	A	0.38	1	0.918
rs2523453	-3285	G	0.38	1	0.918
rs2544525	-3259	C	0.38	1	0.918
rs2523452	-2870	G	0.34	0.953	0.832
rs2596538	-2778	A	0.34	0.953	0.832
rs2844522	-2710	C	0.34	0.953	0.832

Note: Direct DNA sequence of 5-kb promoter region of MICA from 50 HCV-HCC subjects. D' and r^2 were calculated by comparing the genotypes of these SNPs to the marker SNP rs2596542 by Haploview. A1, minor allele; [a] Relative position to exon 1 of the *MICA* gene.

Table 3 Association of SNP rs2596542 and SNP rs2596538 with HCV-induced HCC

SNP ID	Relative position [a]	A1	OR	*P* value
rs2596542	-4815	A	1.339	2.46×10^{-5}
rs2596538	-2778	A	1.343	1.82×10^{-5}

Note: Genotype data of 721 HCV-HCC cases and 5,486 HCV-negative controls were imputed using 1000 genomes as reference. A1, risk allele; OR, odds ratio for the risk allele calculated by considering the protective allele as a reference. [a] Relative position to exon 1 of the *MICA* gene.

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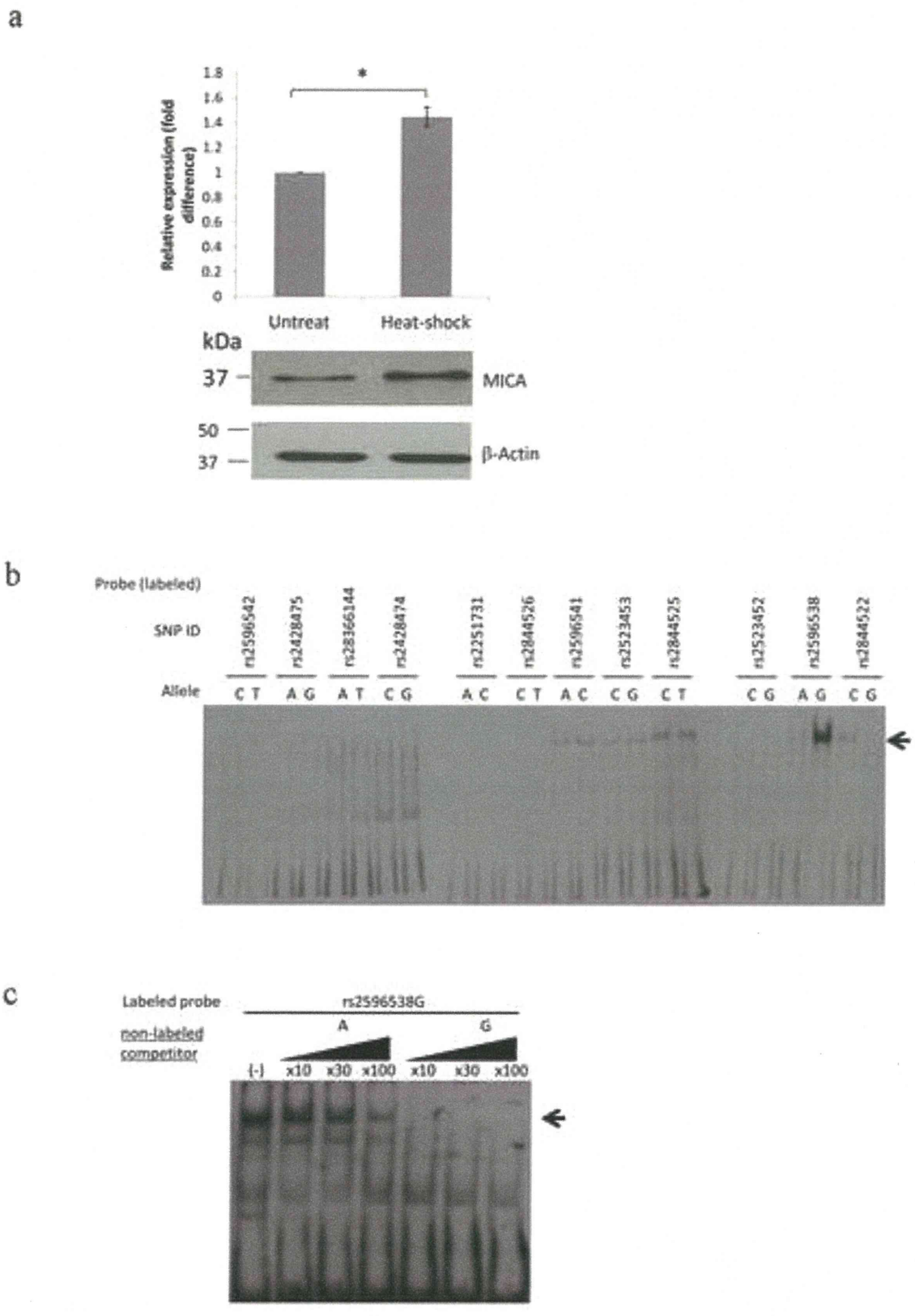

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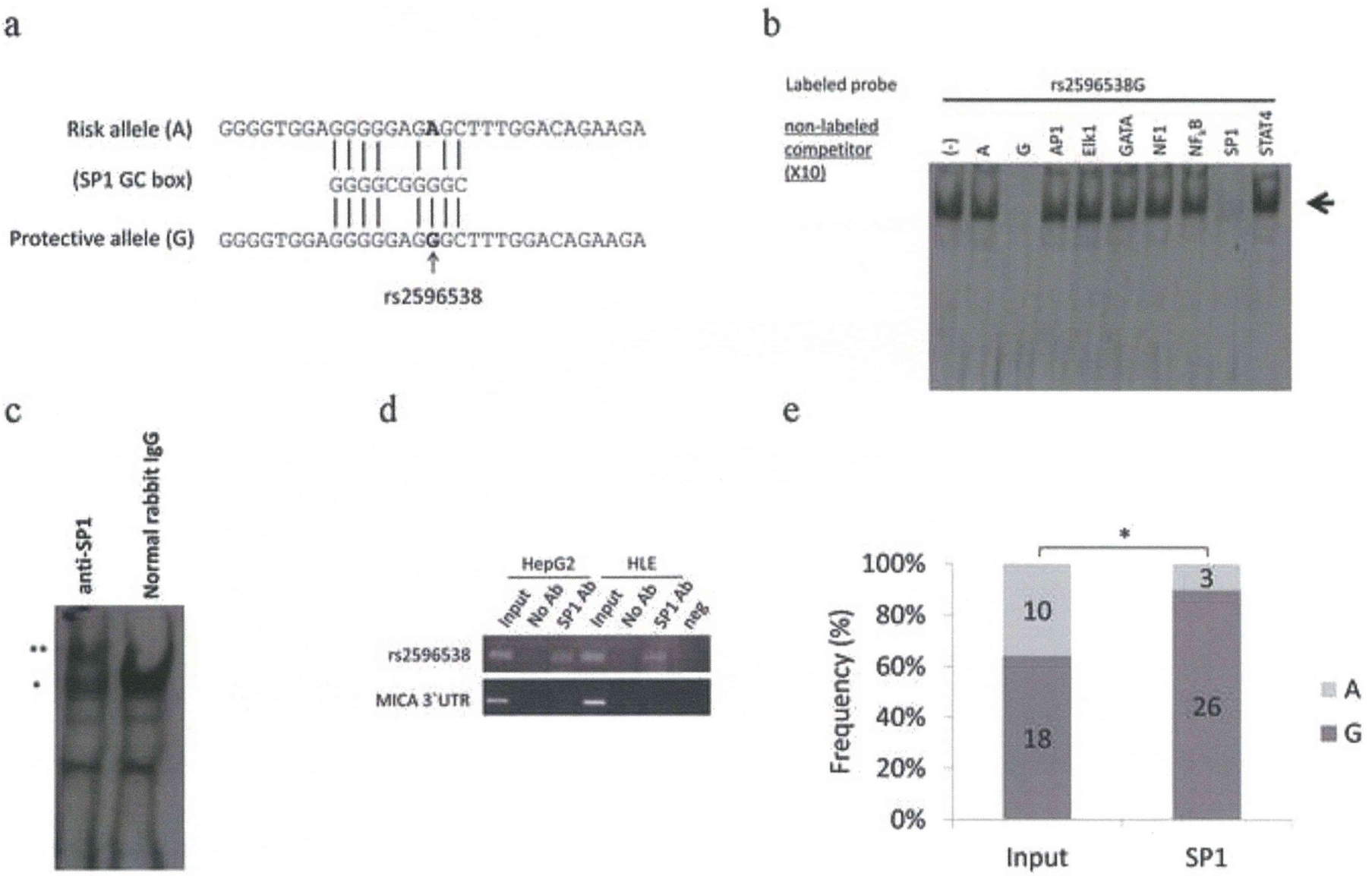

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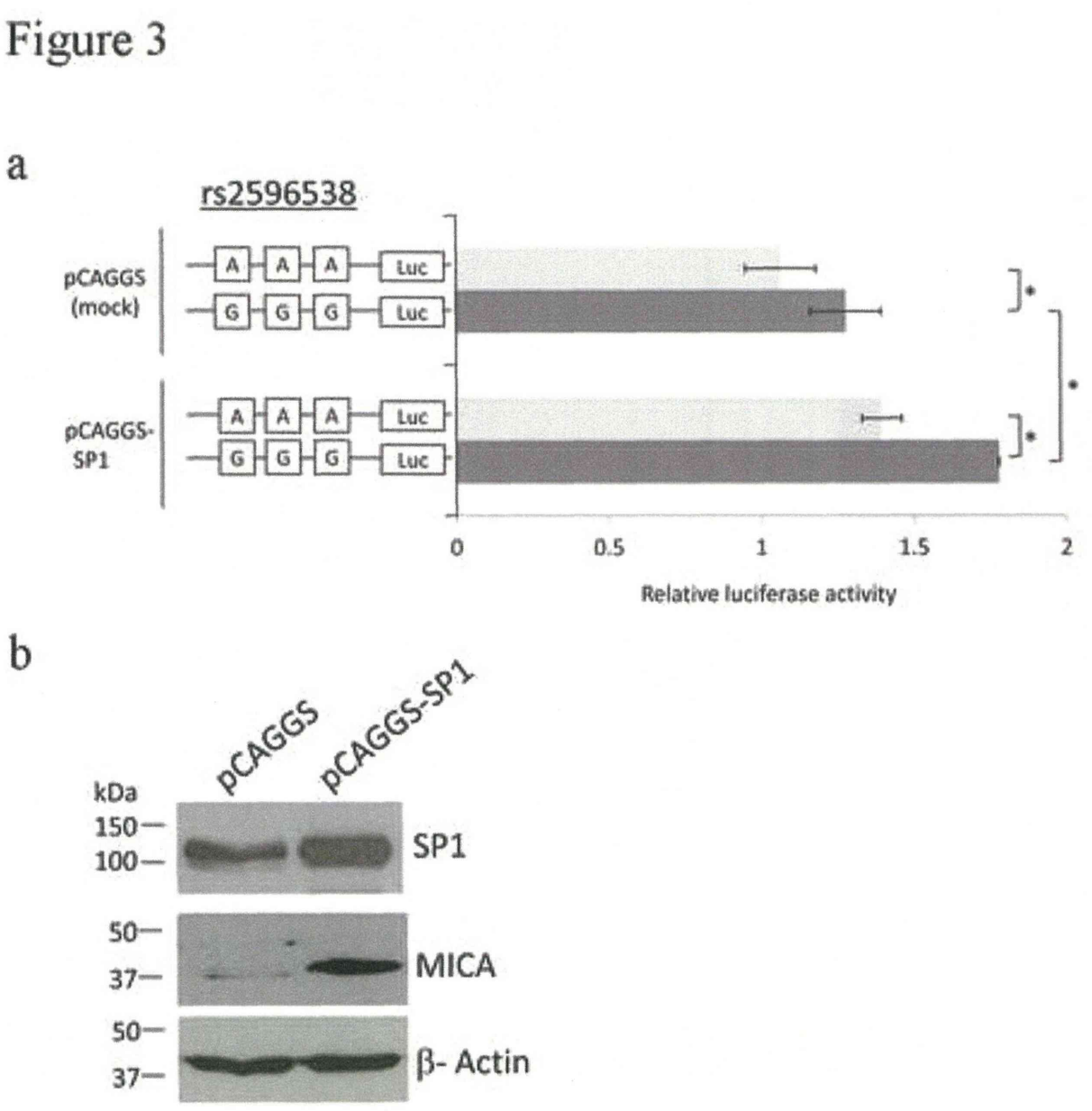

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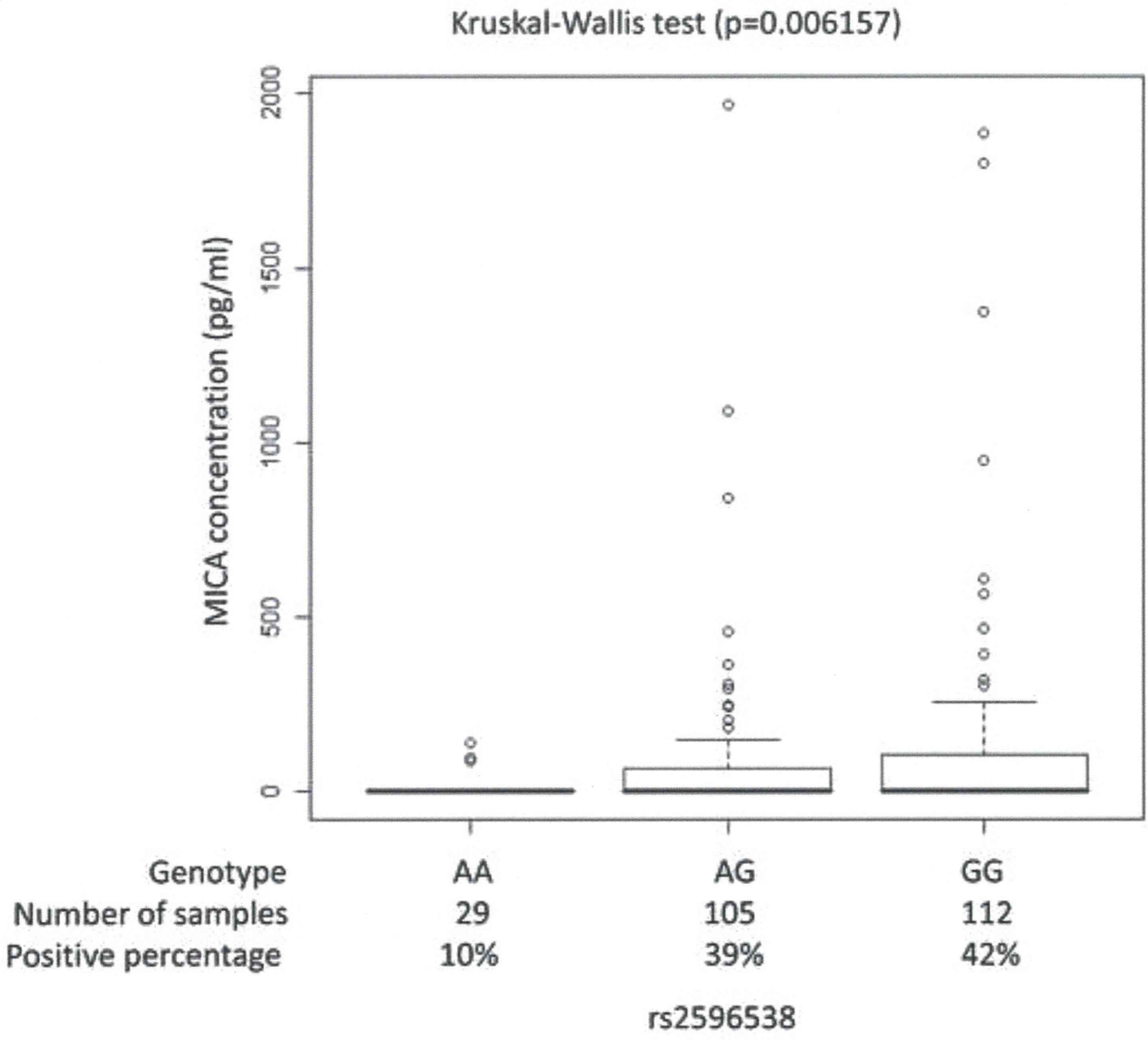

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