Lung Cancer-Associated SNP at the Beginning of Mouse *K-Ras* Gene Intron 2 is Essential for Transcription Factor Binding

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 141, No. 6, pp. 681-684, June, 2006
Original article submitted July 11, 2005

The C→T substitution in position 311 n. p. of *K-ras* gene intron 2 in mice resistant to lung cancer (*M. spretus*) attenuates NF-Y transcription factor binding site in comparison with sensitive ICR mice (*M. musculus*). Appreciable differences between ICR and *M. spretus* in general pattern of binding of nuclear proteins to *K-ras* gene DNA within 248-332 n. p. fragment are demonstrated.

Key Words: *K-ras gene; pulmonocarcinogenesis*

Predisposition of inbred mice to lung cancer is determined by three genetic loci, named Pas (pulmonary adenoma susceptibility) [9]. Pas1 locus on chromosome 6 includes *K-ras* gene [5,10]. Study of *K-ras* gene revealed a correlation between susceptibility of inbred mice (*M. musculus*) to pulmonary adenomas and single nucleotide polymorphism (SNP) in positions 288 and 296 n. p. in the beginning of intron 2 [2,4]. Susceptible mice have nucleotides C and A in these positions, respectively, while resistant mice have G and C nucleotides. We previously showed that "susceptible" CA variant of *K-ras* gene intron 2 SNP corresponds to GATA-6 transcription factor binding site, while in the "resistant" GC variant this site is destroyed [1]. Since GATA-6 is the key factor of lung tissue differentiation and regulation of gene expression in the lungs [8,11], we hypothesized that differences in the binding of this factor to different allele states of *K-ras* gene intron 2 are essential for the formation of lung tumorigenesis-sensitive or resistant phenotype [1].

However, detection of sensitive CA variant of *K-ras* gene intron 2 SNP in *M. spretus* resistant to lung tumorigenesis [10] raised doubt about participation of this polymorphism in the predisposition to carcinogenesis in the lungs. On the other hand, *M. spretus* have one more mononucleotide substitution C (*M. musculus*)→T (*M. spretus*) in position 311 n. p. in the immediate vicinity of SNP in positions 288 and 296 n. p. responsible for the appearance or disappearance of GATA-6 binding site. We therefore hypothesized that this substitution could modify binding site for some transcription factor capable of forming a composition regulatory element together with GATA-6 binding site and be associated with the resistance to lung cancer in *M. spretus*. This study was aimed at verification of this hypothesis.

MATERIALS AND METHODS

Oligonucleotides corresponding to both chains in the region from 278 to 307 n. p. of mouse *K-ras* gene intron 2 and reproducing sensitive CA variant of SNP 5'-cagtGTGCAAGAAA**C**TCCACTT**A**TC ATGAGAGCT-3' and region from 297 to 326 n. p. in *M. musculus* and *M. spretus* mice: MUS 5'-cagtTCATGAGAGCTCAC**C**ACAGAGAAAGAAAGT-3'; SPRET 5'-cagtTCATGAGAGCTCAC**T**ACAGA GAAAGAAAGT-3' (SNP are underlined), and to

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the NF-Y transcription factor binding site 5'-cagtAGACCGTACGTGATTGGTTAATCTCTT-3' (small letters denote the added terminals) were synthesized as described previously [7]. After annealing the oligonucleotides were labeled with [α-^{32}P]dATP using Klenov's fragment.

KE fragments were obtained from genome DNA using polymerase chain reaction with primers KE-Up 5'-GGGTGTTAGGGAACCATAGGTGCAAG-3' and KE-Low 5'-GCACGGATGGCATCTTGG ACC-3' and labeled with [γ-^{32}P]dATP using T4 polynucleotidase. Genome DNA was isolated by phenol chloroform extraction [3].

Extracts of lung cell nuclei from ICR mouse (Institute of Cytology and Genetics) were obtained as described earlier [6] with some modifications [13]. The reaction mixture (16 μl) contained: 1 ng labeled oligonucleotide, 25 mM HEPES (pH 7.8), 80 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM dithiotreitol, 10% glycerol, and 2 μg nuclear extract protein preincubated with sonicated DNA from salmon semen (1 μg/7 μg protein) for 10 min at 0°C. After 15-min incubation at 14°C the mixture was analyzed by electrophoresis in 5% PAAG in 0.5-fold TBE buffer (89 mM Tris borate, 89 mM H_3BO_3, 2 mM EDTA). In tests with antibodies to NF-YA (Santa Cruz Biotechnology, Inc.) the nuclear extract was incubated (15 min at 0°C) with salmon semen DNA and 1 μl antibodies simultaneously.

RESULTS

Gel retardation assay showed that mononucleotide substitution C (MUS oligonucleotide)→T (SPRET oligonucleotide) in *K-ras* gene intron 2 position 311 n. p. attenuates the least mobile retardation band (Fig. 1, *a*), i.e. SNP characteristic of *M. spretus* deteriorates binding site for some transcription factor. Computer analysis of nucleotide sequence using TESS software [14] chose NF1, LKLF, and NF-Y transcription factors as candidates for binding to this site. However, the results of competition analysis and antibody binding showed that the upper retardation band was formed by NF-Y transcription factor (Fig. 1). Thus, the presence of T in position 311 n. p. in *M. spretus* leads to attenuation of NF-Y binding site in comparison to that in *M. musculus*.

Close location of previously detected GATA-6 binding site [1] and NF-Y site in *K-ras* gene intron 2 suggests that they form a more intricate regulatory unit, composition element. This hypothesis is confirmed by detection of similar regulatory units formed by NF-Y and GATA-6 binding sites in other genes [12]. In order to study tentative composition element with genome DNA of ICR and *M. spretus*

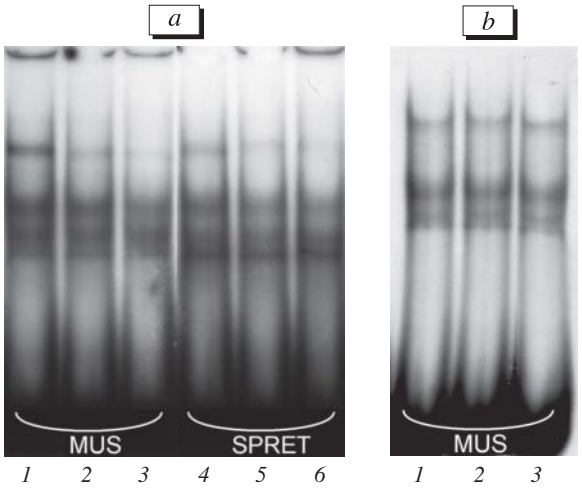

Fig. 1. Effect of SNP in position 311 n. p. of *K-ras* gene intron 2 on NF-Y binding. *a*: *1*, *4*) binding to lung cell nuclear extract proteins without competitive oligonucleotides; *2*, *5*) in the presence of a 50-fold excess of oligonucleotide corresponding to NF-Y binding site; *3*, *6*) binding to the extract in the presence of antibodies to NF-Y. *b*: *1*) binding to lung cell nuclear extract proteins without competitive oligonucleotides; *2*) in the presence of a 50-fold excess of oligonucleotide corresponding to LKLF binding site; *3*) in the presence of a 50-fold excess of oligonucleotide corresponding to NF1 binding site.

mice, 85 n. p. sequences KE-ICR and KE-spret corresponding to 248-332 n. p. region of *K-ras* gene intron 2 were amplified. Gel retardation assay showed that the pattern of binding of lung nuclear proteins to KE-spret fragment differs from that for KE-ICR fragment by the absence of complex *2* and enhanced complex *3* (Fig. 2). Hence, SNP in position 311 n. p. distinguishing these two sequences can cardinally modify the binding patterm of nuclear extract proteins to the studied region. This confirm our hypothesis that NF-Y binding site modified by this mononucleotide substitution is a component of a more complex regulatory structure.

Since experiments with short fragments of *K-ras* gene intron 2 (297/326 n. p.; Fig. 1) demonstrated attenuation of NF-Y site for *M. spretus*, and band *2* is absent in the binding pattern of long fragments (248/332 n. p.) in *M. spretus* (Fig. 2), we can hypothesize that this band represents a complex of the fragment with NF-Y. However, addition of a 100-fold excess of oligonucleotide containing NF-Y binding site into the reaction mixture did not change this band, while band *3* virtually disappeared. We therefore assumed the existence of a more potent binding site for this transcription factor for long fragments. This extra NF-Y site was found in oligonucleotide CA (278/307 n. p., Fig. 3), where it was overlaps GATA-6 binding site [1]. Since addition of antibodies to GATA-6 [1] and NF-Y (Fig. 3) led to complete disappearance of the complex, binding of these transcription factors to the

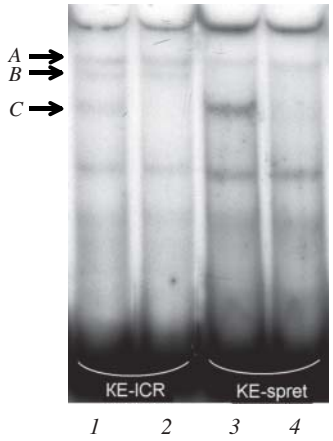

Fig. 2. Binding of lung extract proteins to *K-ras* gene intron 2 fragments (248-332 n. p.) from ICR and *M. spretus* mice. *1*, *3*) binding of KE-ICR and KE-spret fragments to lung cell nuclear extract proteins without competitive oligonucleotides; *2*, *4*) in the presence of a 100-fold excess of oligonucleotide corresponding to NF-Y binding site. Arrows *A*, *B*: complexes with heretofore unidentified proteins; *C*: with NF-Y.

278-307 n. p. region of mouse *K-ras* gene intron 2 can be mutually dependent.

Hence, a set of SNP including C (288 n. p.), A (296 n. p.), and C (311 n. p.) in *K-ras* gene intron 2 in mice susceptible to lung cancer (*M. musculus*) forms a potential composition element including two binding sites for NF-Y and one GATA-6 binding site. Mononucleotide substitutions characteristic of resistant *M. musculus* mice (G 288 n. p. and C 296 n. p.) or *M. spretus* (T 311 n. p.) eliminate different components of this composition element: GATA-6 site in *M. musculus* [1] and NF-Y site in *M. spretus*. Transcription factors binding to the detected sites most likely form an intricate system of interactions (synergic and antagonistic) with each other and with other proteins, which can react with this region. It seems that modifications in the binding of individual transcription factors in this regulatory unit can lead to significant changes in these interactions, which is indirectly seen in the relationship between different SNP of the studied region of *K-ras* gene intron 2 and mouse sensitivity/resistance to pulmonocarcinogenesis.

The study was supported by the Russian Foundation for Basic Research (grant No. 04-04-48136).

Fig. 3. Identification of binding site for NF-Y transcription factor in CA oligonucleotide. *1*) binding of CA oligonucleotide to lung cell nuclear extract proteins without competitive oligonucleotides; *2*) binding to the extract in the presence of antibodies to NF-Y; *3*) in the presence of a 50-fold excess of oligonucleotide corresponding to NF-Y binding site. Arrow: oligonucleotide complex with NF-Y.

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