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SHORT COMMUNICATION

A polymorphism in the AT-hook motif of the transcriptional regulator *AKNA* is a risk factor for cervical cancer

Guillermo Perales, Ana I. Burguete-García, Jisela Dimas, Margarita Bahena-Román, Víctor H. Bermúdez-Morales, José Moreno, and Vicente Madrid-Marina

Instituto Nacional de Salud Pública, CISEI, Division of Chronic Infections and Cancer, Cuernavaca, Morelos, Mexico

Abstract

The *AKNA* gene is part of the 9q32 susceptibility locus for cervical cancer. A single-nucleotide polymorphism at codon 1119 of *AKNA*, yields a biologically relevant amino acid change (R1119Q) at the DNA binding AT-hook motif. Genotype frequencies in 97 allele pairs were: R/R = 0.597, R/Q = 0.278, Q/Q = 0.123. Q/Q homozygosity was present in 8.33% of healthy controls, 16.67% of patients with cervical intraepithelial neoplasia and 75% of cervical cancer patients. These differences are highly significant for the presence of Q/Q in cervical cancer ($p = 0.01$, odds ratio 3.66, 95% confidence interval 1.35–9.94). Therefore, *AKNA* appears to be an important genetic factor associated with the risk cervical cancer.

Keywords: *Cervical cancer; HPV; AKNA; AT-hook motif; SNPs; risk factor; RFLP*

Introduction

Cervical cancer (CC) is the second-largest cause of death by cancer among women around the world, claiming nearly 300 000 victims annually. The persistent infection with high-risk human papilloma virus (HPV) is the primary risk factor for CC (Zur Hausen 1996, Walboomers et al. 1999). The susceptibility of a woman to develop CC is largely attributed to the type of HPV infecting the cervix. Moreover genetic factors can affect HPV infection and modulate the transformation process. Thus, genetic variability of both viral and host factors are involved in the susceptibility and/or resistance to CC. Single-nucleotide polymorphisms (SNPs) are the most common types of genetic variations in the eukaryote genome (International SNP Map working Group 2001). Of the more than 4 million human SNPs mapped so far and deposited in public and private databases, many have been shown to be associated with susceptibility to a wide variety of diseases, although only 3% of them are located within coding regions (Ford et al. 2000). There is increasing evidence for a role of host factors in susceptibility and/or resistance to CC (Calhoun E et al. 2002). Thus, polymorphisms in tumour necrosis factor (TNF) (Kirkpatrick et al. 2004, Wilson et al. 1997), matrix metalloproteinase (MMP)-1, TNF receptor superfamily member 6 (FAS or CD95), promoter regions (Lai et al. 2003), p53 codon 72 (Makni et al. 2000, Dokianakis & Spandidos 2000), p21 codon 31, and interferon regulatory factor-1 (IRF-1) intron 6, have recently been shown to be associated with susceptibility to CC development. Some of these mutations differ in distinct geographical areas (Tenti et al. 2000). Additional host factors studied in cervical tumours include genes that code for proteins involved in a number of processes, ranging from extracellular maintenance and membrane signal transduction to cell cycle regulation. Therefore, it is not surprising that additional polymorphisms could also participate as risk factors for CC.

It has been found that 9q32 contains the susceptibility locus for CC and some of these are candidate genes, potentially involved in the genetic predisposition to this disease; among these genes is *AKNA*, which encodes a

The first two authors contributed equally to the article
Address for Correspondence: Vicente Madrid Marina, Division of Chronic Infections and Cancer, National Institute of Public Health, Av. Universidad 655, Cuernavaca Morelos, Mexico, CP 62508. Tel.: (+52-777) 329-30-00, ext. 2201. Fax: (+52-777) 317-54-85. E-mail: vmarina@correo.insp.mx

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recently discovered transcriptional factor involved in lymphocyte maturation and upregulation of signalling molecules, such as CD40 and CD40L (Engelmark et al. 2006, Siddiqa et al. 2001, Sims-Mourtada et al. 2005). Although the precise molecular mechanisms of AKNA function have not been defined, they could depend, at least in part, through its AT-hook DNA binding motif, which could mediate DNA bending and chromatin rearrangement (Cairns et al. 1999).

Although functional data concerning AKNA are scarce, sequences of *AKNA* genes deposited in the GenBank databases are increasing. SNP analysis in the Genecard site (http://www.genecards.org/) at the Weizmann Institute of Science reveals 313 SNPs for all *AKNA* genes uploaded, but only 11 of them are coding non- synonyms. Among them, *AKNA* SNP (rs3748178) involving the transition g/a, at nucleotide 114189600(-) of chromosome 9 (accession no. AK024431) (Ota et al. 2004), appears to be functionally relevant. This mutation produces an R to Q amino acid change at codon 1119 (protein accession NP_110394). Such R/Q mutation occurs at an important AT-hook DNA binding motif within the highly conserved core GRP (Aravind & Landsman 1998) (codons 1118–1120), producing instead a GQP core motif, which lacks the positive charge of the arginine side chain and, thus, potentially affecting its DNA-binding capacity. The AT-hook motif interacts directly with the minor groove of DNA in AT-rich regions (Huth et al. 1997).

Therefore, we decided to investigate the frequency of arginine (R)–glutamine (Q) alleles as potential risk factors for cervical cancer associated to HPV. Our data show that the presence of the allele homozygote (g/g) R/R or heterozygote (g/a) R/Q are not associated with CC, whereas the allele homozygote (a/a) Q/Q appears to be strongly associated with a high degree of CC.

Materials and methods

Clinical specimens

The selection criteria for the study were HPV16/18-positive cervical lesions ranging from cervical intraepithelial neoplasia (CIN) 1 to CC. The study material consisted of 47 HPV-positive biopsies from Mexican women diagnosed with CIN ($n=21$) or CC ($n=26$). Cases were collected between 1998 and 2003 in general hospitals from the central zone of Mexico. Genomic DNA from controls was obtained through cytobrush of samples of apparently healthy women ($n=50$) without cervical lesions and with HPV-negative status. Informed consent was obtained from all the subjects included in the study, which was approved by the Ethics Committee of the National Institute of Public Health.

DNA template

The *AKNA* gene was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) from peripheral blood mononuclear cells of a healthy individual and was cloned in pCR-TOPOII vector (Invitrogen, San Diego, CA, USA) to yield the pCRTAK plasmid. The fragment corresponding to the AT-hook motif was sequenced and was identical to gene NM_030767 (GenBank accession no.) (allele R). The plasmid pBSFLJ00020, containing the *AKNA* gene with a SNP g/a at the codon 1119 (allele Q) of the AT-hook motif (GenBank accession no. AK024431) was kindly donated by Dr Takahiro Nagase from the Kasuza DNA Research Institute, Chiba, Japan (Ota et al. 2004). Genomic DNA was obtained from biopsy samples or from control cytobrush samples by means of a DNA GenomicPrep, Cell and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech Inc., Amersham, UK). The HPV type was determined from patients' biopsies according to the described technique (Yoshikawa et al. 1991, Bernard et al. 1994).

Polymerase chain reaction of AT/hook motif

The *AKNA* exon 11-specific PCR was developed with primers F-GRP 5′CCA GGG GAT CCG TGA GCT GCA AGA AG 3′, and B-GRP 5′CTG GAT CCA TGG GAG CCC CAG GTG 3′, resulting in a 177-bp amplicon spanning mRNA nucleotides 3384–3560 (GenBank accession no. NM_030767). Plasmid or genomic DNA was used as template for PCR reactions under the following conditions: 100 ng of DNA, 25 µl of 10x PCR buffer, 2.5 mM dNTP, 2.5 mM $MgCl_2$, 50 pmol of each primer and 0.1 µl of *Pfu* polymerase in 25 µl final volume. Denaturation cycle for 5 min at 94°C, then 30 cycles of: denaturation at 94°C for 30 s, annealing at 72°C for 30 s, and elongation at 72°C for 30 s and, finally, one cycle at 72°C for 10 min. PCR reactions were carried out in an Applied Biosystems Gene Amp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Restriction fragment length polymorphism analysis

Because the mutation at nucleotide 114189600(-) in chromosome 9 abolishes a specific site for the enzyme *Eag*I, samples showing differential migration by single-strand conformational polymorphism (SSCP) were restricted with this enzyme to confirm the presence of the specific SNP. Then, samples were separated in 12% PAGE with TAE buffer for 2.5 h and silver stained.

Statistical analysis

Analysis of the information was achieved by comparing the base measurements of age between samples

belonging to each group: patients with CC or CIN and healthy controls. Genotype frequencies were assigned by means of the Fisher's exact test. The Hardy–Weinberg equilibrium between genotypes was evaluated by a χ^2 test. A multinomial logistic regression model was constructed to evaluate the possible specific influence of the *AKNA* genotype on CC and CIN. All tests were two-sided, and $p < 0.05$ was considered statistically significant. All analyses were performed using STATA, version 9.1 for Windows.

Results

Distribution of codon 1119 SNP phenotypes

AKNA is one of the genes found within the 9q32 CC susceptibility locus, which could be involved in the genetic predisposition to this disease (Engelmark et al. 2006). Initially we examined the *AKNA* alleles at codon 1119 within the AT-hook DNA binding motif of *AKNA* corresponding to SNP (rs3748178). PCR fragments from genomic DNA sequence 3384–3560 of *AKNA* were examined by restriction fragment length polymorphism (RFLP) analysis and compared with unique 177-bp PCR fragments from pCRTAK and pBS-FLJ00020 plasmids, which contain the reference R and Q alleles, respectively (Figure 1A, lines 2 and 3). Because the transition A/G at codon 1119 implies the loss of the *Eag*I restriction site, to ensure that the SNP under investigation occurred specifically at the codon 1119, all PCR samples ($n = 97$) were subjected to *Eag*I restriction analysis. Allele R maintains the *Eag*I restriction site and yields 130-bp (Figure 1B, line 2) and 47-bp fragments (not seen in the figure). On the other hand, allele Q loses the *Eag*I restriction site and remains as a 177-bp fragment (Figure 1B, lane 3). Finally, heterozygotes have a combined pattern with both 177-bp and 130-bp fragments as seen in Figure 1B, lanes 4 and 5.

Furthermore, the distribution of *AKNA* genotypes in the whole group of 97 women includes 58 R/R homozygotes (59%), 12 Q/Q homozygotes (13%) and 27 were R/Q heterozygotes (28%). In this manner, genotype frequencies in the population (194 chromosomes) were: a/a = 0.123, a/g = 0.278 and g/g = 0.597. The frequencies of the alleles are a = 0.262 and g = 0.737, meaning that our population is in Hardy–Weinberg equilibrium. Likewise provided that our study is a design of cases and controls, the χ^2 test for the Hardy–Weinberg equilibrium between the genotypes shows that the genotypes are in equilibrium in the healthy woman group, ruling out the possibility of bias of selection (Table 1).

Gln/Gln homozygosity is strongly associated with cervical cancer

The characteristics of the studied population are shown in Table 1, agreement of the assigned groups (patients with CC, CIN and healthy controls), statistically significant differences were detected in the age variable and, hence, were considered in the analysis as potential confounders. We found 8.33% of homozygotic pattern Q/Q in the healthy group ($n = 50$), 16.67% in the CIN patients ($n = 21$) and in 75% of the CC patients ($n = 26$). These results show a strong increase (almost x 9) of the Q/Q homozygosity in the cancer group.

Then we evaluated the distribution of the different genotypes in the three study groups obtaining statistically significant differences by Fisher's exact test.

Using a bivariate analysis with a model of multinomial logistic regression, coefficients of regression were

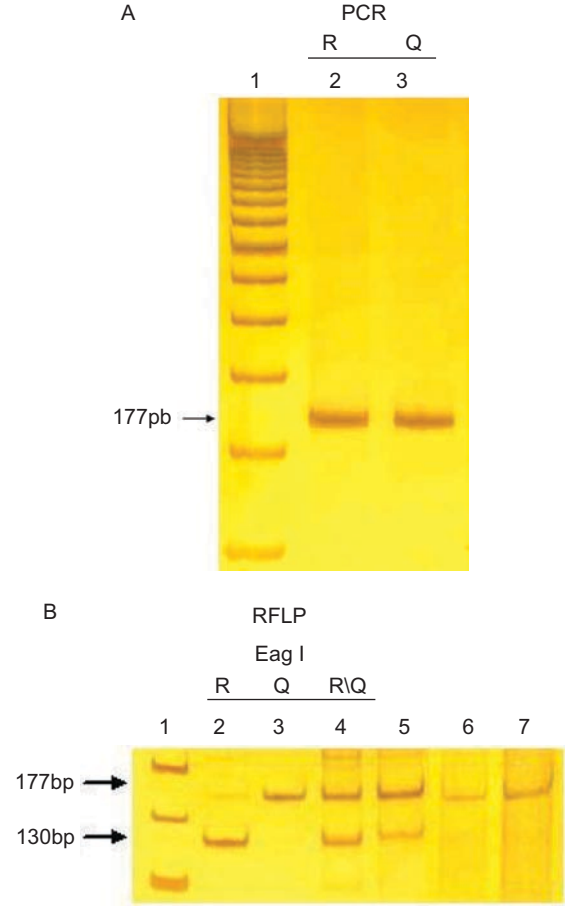

Figure 1. Polymerase chain reaction (PCR) of the AT-hook region of *AKNA*. (A) Amplification by PCR, of a fragment of *AKNA* gene that include AT-hook motif. Line 1, MW 50 bp; line 2, 177-bp fragment amplification by PCR from plasmid DNA control for arginine (R) and from plasmid DNA control for glutamine (Q), line 3. (B) Restriction fragment length polymorphism (RFLP) analysis of *AKNA* 1119 codon polymorphisms. Restriction analysis of the PCR product with the *Eag*I enzyme. The PCR products were restricted with *Eag*I enzyme. Lane 1, MW 50 bp; lane 2, *AKNA* gene allele R; lane 3, *AKNA* gene allele Q; lanes 4–7, representative samples from patients and control subjects. Lane 4, CIN patient showing heterozygotic status R/Q; lane 5, a healthy woman showing heterozygotic status R/Q; lane 6, cervical cancer patient showing homozygotic status Q/Q; lane 7, a patient showing homozygotic status Q/Q.

calculated, with respective intervals of confidence to 95% (IC 95%), including in the multiple model the age variable as a potential confounder.

The Q/Q polymorphism of the gene *AKNA*, confers a nearly fourfold higher risk of developing CC regardless of age (Table 2). These data provide evidence that the transcriptional regulator *AKNA* is a potential genetic factor associated with CC development, at least in Mexicans and probably in other populations.

In short, our study shows the presence of R to Q SNP in the genome and the frequency of the homozygous phenotype Q/Q (0.12) indicates that this allele should be considered a less common variant of *AKNA*. In addition, a bivariate analysis with a model of multinomial logistic regression of the results provides evidence that *AKNA* Q/Q homozygosis is a risk factor for CC associated with HPV.

Discussion

In the current studies we examined the frequency of two variants of SNP (rs3748178) of *AKNA*, belonging to the 9q32 susceptibility site for CC. This SNP is expected to be biologically relevant because it produces a R to Q change in the AT-hook motif of *AKNA*, which is involved in DNA binding. For this purpose, we standardized a RFLP technique to genotype *AKNA* allele Arg and Gln variants in the AT-hook motif of the AKNA protein. The frequency of these polymorphisms was examined in three groups of women: healthy women, patients with cervical lesions and patients with CC. The results indicate that the homozygotic Q/Q status at the aforementioned gene region is strongly associated with the risk of CC.

AT-hook motifs mediate binding of proteins to DNA, preferentially to the minor groove of stretches of AT-rich sequences. AT-hook-containing proteins are of high relevance to biology, as they mediate several processes through binding to DNA, which include the control of chromatin structure and transcriptional regulation. Thus, it is not unexpected that polymorphisms in the AT-hook of transcriptional regulators could have a strong influence on disease susceptibility. Arginine residues in AT-hook motifs are responsible of DNA binding as it was shown for the herpes virus Saimiri open reading frame 50 (ORF50) (Walters et al. 2004). Although for this particular protein, exchange of a single arginine to alanine in the core GRP is not sufficient to ablate DNA binding and transcriptional activity. However, Sgarra et al. (2006) found that methylation of residue R of the GRP core and/or the preceding R, in the AT-hook motif of the high mobility group (HMG) A1 protein strongly affects DNA binding of this important protein. Such methylation could be comparable to the R to Q polymorphism of AKNA we have examined here. Although we do not know yet the functional consequences of this polymorphism, its association to CC raises the possibility that the AKNA protein is controlling some as yet unknown proteins involved in the control of CC susceptibility.

AKNA protein has been implicated in the upregulation of immunoregulatory molecules, such as CD40 and CD154 (Siddiqa et al. 2001), but a direct role in the process of the infection HPV and/or tumour eradication has not been reported to date. Nevertheless, Engelmark et al. (2006) using a genome-wide scan approach identified three susceptibility loci for cervical carcinoma, one of these loci, 9q32, which has the highest multipoint LOD score, includes the *AKNA* gene. The data reported here, support the importance of the genomic region where the *AKNA* gene is located. Although AKNA has several isoforms due to both alternate splicing and varying translation initiation sites, the AT-hook AKNA motif studied in this work is present in all AKNA isoforms reported except in the C1 and C2 isoforms (Sims-Mourtada et al. 2005). Therefore, our observation is valid for most of the isoforms of AKNA and further work will aim to define in a more precise manner which of those isoforms is or are responsible for CC risk.

In conclusion, the frequencies observed of genotype Q/Q of *AKNA* in this group of 194 chromosomes studied,

Table 1. Characteristics of subjects from the population studied, allelic frequencies and Hardy-Weinberg equilibrium.

	Diagnosis			
	CxCA ($n = 26$)	CIN ($n = 21$)	Controls (n = 50)	*p*-Value
Age (SD)[a]	46 (10.22)[a]	34 (11.48)[a]	35 (13.76)[a]	**0.0005**
AKNA (%)[b]				
RR	15.52	27.59	56.90	
RQ	29.63	11.11	59.26	**0.000**
QQ	75	16.67	8.33	
R	50	83	82	
Q	50	16	18	
HWE_C			1.0000	

Test: [a]equality of populations (Kruskal–Wallis test); [b]Fisher's exact test.

CxCA, cervical cancer; CIN, cervical intraepithelial neoplasia, HWE_C, Hardy–Weinberg equilibrium in healthy controls

Table 2. Risk of illness associated to genotype Q/Q.

	OR_c	95% CI	*p*-Value	OR_a	95% CI	*p*-Value
CxCA	**3.66**	1.35–9.94	**0.01**	**4.22**	1.3–13	**0.01**
CIN	0.60	0.18–1.93	0.39	0.61	0.18–2.0	0.42

CI, confidence interval; CxCA, cervical cancer; CIN, cervical intraepithelial neoplasia. Multinomial logistic regression: OR_c, unadjusted odds ratio; OR_a, adjusted OR by age.

indicate that this (Q1119) is an important variant of gene *AKNA*, at least in the population studied. It is also important to study the effect of the Q mutation in *AKNA* on the DNA binding activity, and understand the physiological condition of *AKNA* homozygote Q/Q in women. It is of high relevance the demonstration of a statistically significant association between a mutation in the *AKNA* gene (that results in a R-Q amino acid change) and susceptibility to CC. This is potentially biologically significant given that the gene is located within a known cervical cancer susceptibility locus and the mutation is in a significant portion of the protein, the AT hook. This observation opens new questions about the AKNA functions. Whether this mutation contributes to an alteration of AKNA protein structure and immune function, and its contribution to CC development is under investigation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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