Asian-Aust. J. Anim. Sci.
Vol. 21, No. 8 : 1103 - 1108
August 2008

www.ajas.info

Induction of Folate Sensitive Chromosomal Fragile Sites by Fudr in Pakistani Lohi Sheep (*Ovis aries*)

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ABSTRACT : An investigation to determine frequency and distribution of folate sensitive chromosomal fragile sites was carried out in a Pakistani breed of Lohi sheep to uncover fragile site phenomena. The means and standard errors of aberrant cell count (AC) and Number of aberrations (NoA) in Lohi sheep were 0.56±0.15 and 0.59±0.16 in the control cultures. FUdR treated cells showed significantly higher ($p<0.001$) AC and NoA means (2.18±0.33 and 2.65±0.50). The sex comparison for the frequency of expression indicated that males had significantly higher number of aberrant cells and total number of aberrations in FUdR cultures than the female group in Lohi sheep. The comparison of control cultures was however, not significantly different between the two groups. The regression analysis of FUdR-induced chromosomal fragility data analysis of the fragility data predicted very low β of 0.325 and 0.412 for AC and NoA respectively. Lohi chromosomes expressed lesions in only 7 and 24 bands in the control and FUdR cultures respectively. The total number of significantly fragile bands in the Lohi genome was only 4. The X-chromosome of the Lohi sheep was highly stable at 5 µg/ml FUdR with no fragile sites. The sex comparison for the distribution of fragile sites across the Lohi genome did not reveal any noticeable differences. (**Key Words :** Folate Sensitive, Chromosomal Fragile, Fudr, Pakistani Lohi, Sheep)

INTRODUCTION

Fragile sites are weak genomic points on chromosomes that are involved in chromosomal rearrangements, deletions and reciprocal translocations and are also correlated with points of evolutionary importance involved in the divergence of different species (Yang and Long, 1993; Ronne, 1995; Ruiz-Herrera, 2005). Similarly they show gaps, breaks and multi-radial figures either spontaneously or upon induction *in-vitro* using different carcinogenic agents such as 5-fluorodeoxyuridine (FUdR), Aphidicoline (Yang and Long, 1993). More recently gene mapping analysis in river buffalo revealed that the closest genes spanning fragile sites are RASA1, CAST, NPR3, C9, OarCP09, PLP and BTK and EDNRB. Any mutations of these genes result in severe phenotypic malformations, immunodeficiency and meat quality in different species (Nicodemo et al., 2007). Folate sensitive fragile site expression seems to appear as a result of heritable defects of DNA methylation along a region normally binding a folding protein involved in chromosome condensation. Impairment of DNA-folding-protein interaction would result. A superimposed folate deficiency, or any condition leading to impaired thymidylate biosynthesis, would promote mis-incorporation into DNA of uracil in place of thymine, thus producing a further loss of methyl groups at the fragile site and eventually precluding a full DNA-folding-protein interaction. A localized collapse of the chromosome structure would follow (Carlos et al., 2005). Of 33 well-defined sheep breeds, Lohi is the most extensively reared breed of sheep in the central parts of the Punjab province of Pakistan. Information on the cytogenetic and molecular genetic aspects of Pakistani sheep breeds is scanty in Pakistan (Steane, 1997). Lohi is reported to have 54,XX and 54XY chromosomal complement with three metacentric pairs and 50 sub-acrocentric or acrocentric pairs (Babar et al., 1991). The X chromosome is the largest subacrocentric and Y is the smallest metacentric chromosome in Lohi sheep (Ali, 1993). Pakistan possesses extensive sheep genetic resources of 26.5 millions heads, representing more than 25 breeds, some of them are at dangerous threshold of extinction. It is therefore the highest time to undertake conservation of these breeds through molecular characterization. More recently extensive studies are reported both in Pakistani and Indian breeds to genetically

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Received August 27, 2007; Accepted November 25, 2007

characterize and conserve (*Ovis aries*) as a valuable and diverse animal genetic resource (Bhatia and Arora, 2005; Hirbo et al., 2006; Girish et al., 2007). This is the first ever fragile site study in any Pakistani domestic livestock species. This study was undertaken to unmask and determine the frequency and distribution of autosomal and sex chromosome fragile site loci and their biological significance in Lohi sheep. Fragile sites are also implicated as hot spots of evolutionary significance as well as chromosomal aberrations such as reciprocal translocations (Long, 1997).

MATERIALS AND METHODS

Animals

Haparinised whole blood samples from 20 ewes and 12 rams of the Lohi Breed were taken by jugular venepuncture to set up cultures for chromosomal studies.

Setting up cultures

Blood (1 ml) was mixed with 4 ml RPMI-1640 with 5% foetal calf serum and 1,000 IU Penicillin/Streptomycin supplemented with 0.1 ml L-glutamine and 0.15 ml of Pokweed mitogen under a safety flow cabinet. The culture tubes were then left for 72 h at 37°C. The same cultures were synchronized with 5 μg/ml FUdR (Sigma-F0530) after 48 h for the induction of fragile sites. Blood samples from each animal were grown in three replicates for FUdR induction of folate sensitive fragile sites. The control culture without FUdR was grown separately.

Cell harvesting

After completing the 72 h culture period the samples were left at room temperature for 30 minutes and spun at 50 g for 10 minutes. The cell pellet was gently stirred with a Pasteur pipette. The cell pellet was then suspended in a 0.0168 M hypotonic solution of KCl pre-warmed at 30°C for 15-20 minutes. After 10 minutes of hypotonic treatment 0.1 ml of 10 μg/mlof colcemid was added. This process was completed at an incubating temperature of 28-30°C. After hypotonic/colcemid treatment the cell suspension was spun down at 50 g. The cell pellet was then ready for three runs of fixation with 3:1 methanol acetic acid fixative chilled at -20°C. After discarding the supernatant the cells were ready to be plated.

Slide preparation and Giemsa staining.

Ethanol dipped and dried glass slides were labelled and used to drop 50 μl of cell suspension using a Pasteur pipette from a height of 1-2 ft for each animal. The slides were left on a wet gauze for few seconds while gently washing and blowing the plated cell suspension with fixative solution using a slow air drying technique to obtain better quality metaphase spreads. The slides were then stained using 10% Giemsa-Gurr solution (pH 6.8) by dipping in a coupling jar for 10 minutes. After washing the slides with tap water and blot drying, they were ready for microscopic examination.

Fragile site scoring, G-banding and photography

For each individual animal the control and FUdR treated slides were screened thoroughly with a light microscope to locate a minimum of 50 metaphase spreads. The metaphases on each slide were recorded using an England finder. Aberrant Cell Count (AC) and total number of aberrations (NoA) in each animal were used as the measure of fragile site frequency. The aberrant cell count (AC) was defined as the number of cells with gaps, breaks and deletions whereas number of aberrations (NoA) was defined as the total number of chromosomal lesions in 50 cells both in control and FUdR cultures. Each metaphase found with single or multiple chromosomal lesions was photographed using a manual orthomate camera with black and white technical pan Kodak film. The same slides were then de-stained with 3:1 methanol and acetic acid solution and left overnight. GTG-bands were induced to identify/localize chromosomal gaps and breaks by using 0.3% trypsin solution in 9 ml of Sorensen's buffer. Cytogenetically screened and fragile- site-data recorded slides were treated with this solution for 10-20 seconds to obtain optimal G-bands for localizing fragile sites to individual chromosomal bands. G-band nomenclature of Long (1985) was used as a standard to assign chromosomal lesions to individual chromosomal bands. Due to the small size of chromosome pairs 21-26, they were not included in the study to avoid erroneous fragile site distribution data.

Data analyses

Fragile site frequency data were analysed using the non-parameteric, Mann-Whitney test (Minitab Statistical Software Release 3.11) to compare significance of fragile site expression between control and FUdR treated cells. A multinomial model proposed by Bohm et al. (1995) was used to identify significantly occurring fragile sites in the sheep genome. This model allows analysis of pooled data over groups of individuals to establish non-random site-specific chromosomal breakage with respect to total breakage. It uses a stepwise procedure of fitting a multinomial model that assigns equal probabilities to a "maximal" set of non-fragile sites and unrestricted probabilities to remaining (fragile) sites with significantly higher number of breaks. In order to measuring a goodness of fit, it utilises Pearson's chi-square (x^2) and likelihood-ratio (G^2) test statistics, being most appropriate for a multinomial distribution. This multinomial model for

Table 1. Comparison of the means and standard errors of (AC) and (NoA) at different FUdR levels in Lohi sheep

Variables/FUdR	FUdR 0 µg/ml	FUdR 5 µg/ml	Test statistic
Aberrant cell count (AC)	0.56±0.15	2.18±0.33	$p<0.01$
No. of lesions (NoA)	0.59±0.16	2.65±0.50	$p<0.01$

analysing fragile site data was programmed as an MS-DOS computer programme *FSM Version 995*. (Greenbaum and Dahm, 1995).

RESULTS AND DISCUSSION

Means and standard errors of AC and NoA per animal in the control and FUdR treated cultures from 32 Lohi animals are presented in Table 1. The comparison of the data on AC and NoA between the control and FUdR cultures in the present group of Lohi sheep, revealed a highly significant variation ($p<0.01$).

The comparison of the AC and NoA means within each treatment however, revealed a non-significant variation. Therefore, there were almost as many lesions as the number of aberrant cells both in the control as well as FUdR cultures in Lohi sheep and FUdR did not induce a significantly higher number of chromosomal lesions in aberrant cells. The incidence of folate sensitive chromosomal lesions with FUdR treatment, and their identification as fragile sites in the Lohi breed from Pakistan, substantiates the observation that fragile sites are a regular feature of the mammalian genome (Ruiz-Herrera, 2005). The extent of this cytogenetic damage, however, may vary depending upon different genetic and environmental factors.

The data on spontaneous and FUdR induced lesions was also subjected to linear regression analysis. The regression or prediction equation for AC and NoA in the Lohi sheep was:

$$AC = 0.56+0.325\ FUdR,$$

$$NoA = 0.594+0.412\ FUdR$$

Significant variation in cumulative and per animal in the control and FUdR cultures in the Lohi sheep was in agreement with the findings of similar studies reported elsewhere. The folate sensitive fragile site studies conducted in most animal species have also shown significant increases in the mean number of aberrant cells and the total number of lesions (Stone et al., 1991a, 1991b; Ronne, 1992; Stone and Stephens, 1993; Yang and Long, 1993; Lopez and Arruga, 1996; Nicodemo et al., 2007).

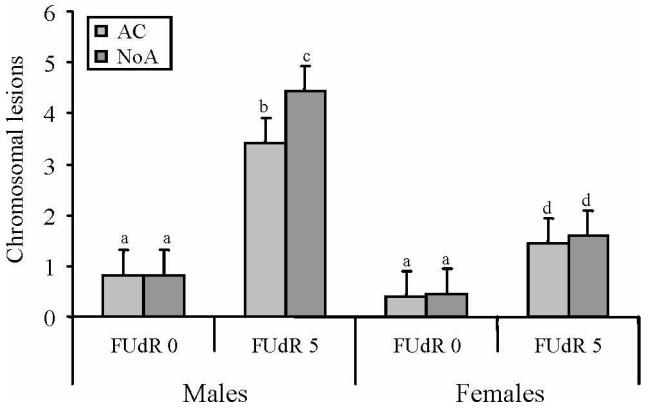

Figure 1. Comparison of Spontaneous and FUdR induced chromosome fragility between Lohi male and female animals. The means with same superscript do not differ significantly from each other.

Gender variation in fragile site expression

The comparison of AC and NoA means between Lohi males and females revealed that the means for AC and NoA were not significantly different in the control cultures. However males were shown to have significantly higher means for both AC and NoA in FUdR cultures (Table 2). This variation between male and female groups of Lohi sheep might be breed/species specific. Breed or species variation may be attributable to differences in DNA replication under FUdR stress between males and females. A significant variation in the number of aberrant cells and total number of lesions between female and male animals was also reported by Yang and Long (1993) in pig chromosomes. This variation was opposite to our finding and females had significantly higher means for AC and NoA. The contrary results obtained by Yang and Long (1993) could be attributed to sample size (2 males vs. 2 females). The present study used a much larger sample size for more reliable conclusions (12 males and 20 ewes). The results of investigations in human chromosomes from 82 healthy males and females however, revealed non-significant variation in the total number of lesions induced by FUdR (Smeets and Merkx, 1990). The findings in two groups of dogs were also shown to be non-significant (Stone et al., 1991a). The results of different studies quoted above are highly variable and might be explained on the basis of different criteria in scoring fragile sites, the

Table 2. Comparison of means and standard errors of AC and NoA at different FUdR levels between Lohi males and females

Parameters	Males		Females		Test statistcs	
FUdR conc.	FUdR 0	FUdR 5	FUdR 0	FUdR 5	FUdR 0	FUdR 5
AC	0.83±0.32	3.42±0.67	0.40±0.15	1.45±0.22	$p>0.05$	$p<0.05$
NoA	0.83±0.32	4.42±1.10	0.45±0.18	1.60±0.27	$p>0.05$	$p<0.05$

Table 3. Chromosomal gaps and breaks mapped to Individual chromosomal G-bands at different FUdR levels in lohi sheep (*Ovis aries*) genome

0 µg/ml		5 µg/ml						FSM995
Bands	Freq.	Bands	Freq.	Bands	Freq.	Bands	Freq.	
3q12	1	1p14	1	5q32	1	14q31	1	2q21*
5q32	1	1p22	1	6q22	1	15q12	1	9q12*
8q12	1	2p21	1	6q24	2	16q14	1	10q14*
9q22	1	2q14	1	8q22	1	18q12	1	
9q24	1	2q21	4	8q24	1	19q14	1	
10q14	1	2q32	2	9q12	3	Xq17	1	
19q14	1	3q12	1	9q22	1	Xq22	1	
		4q17	1	10q12	2	Xq24	1	
				10q14	4			

Bold Italics indicate homologous chromosomes.
* Statistically identified fragile sites by FSM 995 computer version in control and FUdR treated cultures respectively.

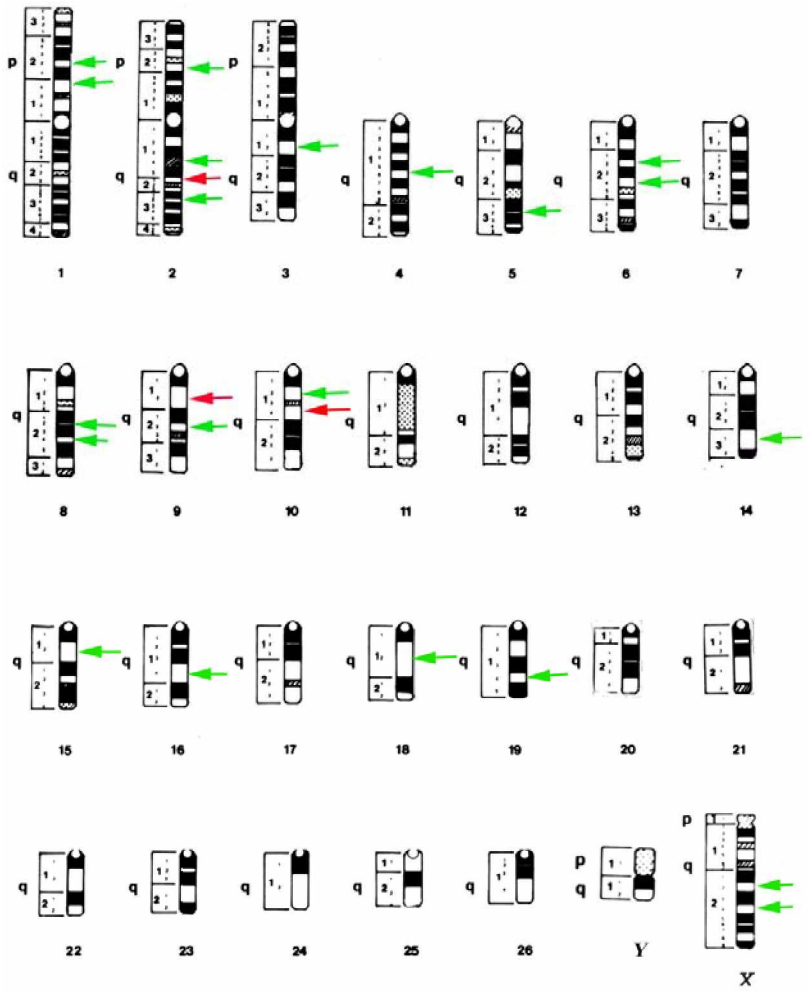

Figure 2. Fragile site map of the Lohi sheep following FUdR treatement. Green arrows = Chromosomal lesions induced by FUdR. Red arrows = Chromosomal lesions induced by FUdR and statistically identified as fragile sites by Multinomail model by Bohm et al. (1995).

inducing agent or the variation owing to breed/species.

Distribution of spontaneous and FUdR induced lesions and identification of fragile sites in the Lohi genome

Chromosomal band data obtained in the control and FUdR cultures showed 7 lesions mapped to individual chromosomal bands in the control cultures. Similarly 36 lesions were mapped to 23 autosomal and 3 X-chromosome bands in FUdR treated cells. Statistical analysis of chromosome band data revealed only three chromosomal bands (2q21, 9q12, and 10q14) to be statistically fragile in the Lohi sheep genome (Table 3). The analysis of reported

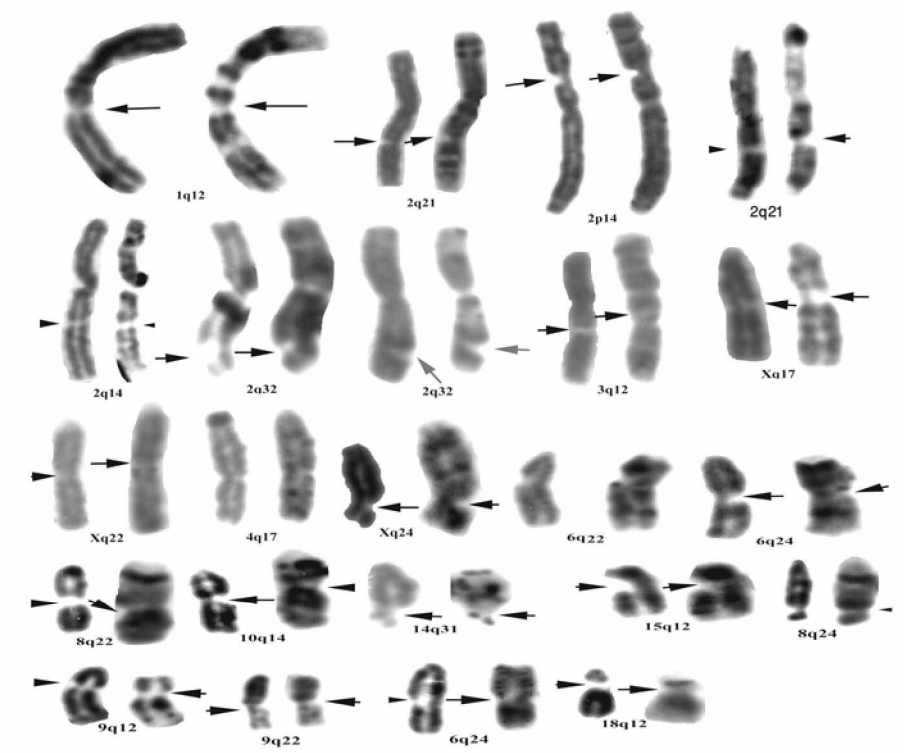

Figure 3. FUdR induced fragile sites mapped to individual chromosomal bands.

chromosomal loci involved in reciprocal translocations in sheep (Long, 1997) did not reveal any correlation with mapped fragile sites in Lohi sheep. The fragile site map data obtained in this study was not large enough to draw any conclusive evidence about the two phenomena.

In addition the quality of G-bands was poor in Lohi chromosomes especially with FUdR treated cells. This was primarily due to exhaustive staining, de-staining and re-staining procedures applied for data recording, coupled with possible antagonism of FUdR treated chromosomes to trypsin treatment. All chromosomal lesions were mapped to light G-bands and chromosomal lesions detected in the control cultures were also mapped in the FUdR treated chromosomes except 8q12. This might be due to fact that many chromosomal lesions escaped identification due to poor quality of G-bands in exhaustively used slides for fragile site scoring or its isolated appearance in control cells may be due to cultural artefact. However, this observation does not necessarily indicate that fragile site expression is a partially random phenomenon. Many workers studied and have reported non-randomness of fragile site expression (Barbi et al., 1984; Hecht, 1985; Bohm et al., 1995).

Distribution comparison of spontaneous and FUdR induced lesions between males and female groups

Comparison of the distribution of chromosomal lesions between Lohi females and males did not reveal any conclusive information, probably due to smallness of the data owing to the remarkable stability to cytogenetic damage both in the control and FUdR cultures of Lohi sheep. An important observation was that the male X chromosome did not show fragility whereas the female cells expressed lesions at Xq17, Xq22 and Xq24. It may be inferred that the male X chromosome was relatively inert in the Lohi sheep. This is also in agreement with our findings in the British counterpart where X-chromosome fragility was mainly expressed by female cells (under publication).

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