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Chromosomal expression and localization of aphidicolin-induced fragile sites in the standard karyotype of river buffalo *(Bubalus bubalis)*

Nicodemo D.[a] · Coppola G.[a] · Pauciullo A.[a] · Cosenza G.[a] · Ramunno L.[a] · Ciotola F.[b] · Peretti V.[c] ·Di Meo G.P.[d] · Iannuzzi L.[d] · Rubes J.[e] · Di Berardino D.[a]

[a]Department of Soil, Plant, Environment and Animal Production Sciences, University of Naples 'Federico II', Portici

[b]University of Catanzaro 'Magna Grecia', Catanzaro

[c]Department of Animal Science and Food Inspection, University of Naples 'Federico II'

[d]National Research Council (CNR), ISPAAM, Laboratory of Animal Cytogenetics and Gene Mapping, Naples (Italy)

[e]Veterinary Research Institute, Brno (Czech Republic)

Corresponding Author

Prof. Dino Di Berardino

Department of Soil, Plant Environment and Animal Production Sciences

University of Naples 'Federico II'

Via Università 133, IT–80055 Portici (NA) (Italy)

telephone: +39 081 253 9265; fax +39 081 776 2886

e-mail: diberard@unina.it

Abstract

The present study reports on the chromosomal expression and localization of aphidicolin-induced fragile sites in the standard karyotype of river buffalo (Bubalus bubalis, 2n = 50) with the aim of establishing a 'fragile site map' of the species. Totally, 400 aphidicolin-induced breakages were analyzed from eight young and clinically healthy animals, four males and four females; these breakages were localized in 106 RBG-negative chromosome bands or at the band-interband regions. The number of breakages per chromosome did not vary statistically 'among' the animals investigated but the differences among individual chromosomes were highly significant thus indicating that the chromosomal distribution of the breakages is not random and appears only partially related to chromosome length. Fragile sites were statistically determined as those chromosomal bands showing three or more breakages. In the river buffalo karyotype, 51 fragile sites were detected and localized on the standardized ideogram of the species. The most fragile bands were as follows: 9q213 with 24 breakages out of 400; 19q21 with 16, 17q21 and inacXq24 with 15, 15q23 with 13 and 13q23 with 12 breaks, respectively. Previous gene mapping analysis in this species has revealed that the closest loci to these fragile sites contain genes such as RASA1 and CAST (9q214), NPR3 and C9 (19q19), PLP and BTK (Xq24-q25), OarCP09(15q24), and EDNRB (13q22) whose mutations are responsible for severe phenotypic malformations and immunodeficiency in humans as well as in mice and meat quality in pigs. Further cytogenetic and molecular studies are needed to fully exploit the biological significance of the fragile sites in karyotype evolution of domestic animals and their relationships with productive and reproductive efficiency of livestock.

Introduction

Fragile sites (FSs) are specific loci that show gaps, breaks or rearrangements in metaphase chromosomes when cells are cultured under conditions that inhibit DNA replication. They are late replicating, evolutionarily conserved 'hot spots' for increased sister chromatid exchanges (SCEs), translocations and deletions and preferred sites for genetic recombination, viral integration and

carcinogenesis. While a conspicuous bulk of research has been conducted in humans, domestic animals have received only little attention. Cattle are the most investigated domestic species (Rodriguez et al., 2002), followed by pig (Ronne, 1995), rabbit (Paulsen and Ronne, 1991) and horse (Ronne, 1992). In river buffalo, Balakrishnan et al. (1988) reported gaps on the X chromosome of anestrus females suggestive of a FS whereas Pires et al. (1998) found FSs on the X chromosome in three breeds of river buffalo, but so far no detailed information is available on the localization of FSs on the karyotype of this species, whose banded karyotypes have been quite extensively characterized (Di Berardino et al., 1981; Di Berardino and Iannuzzi, 1984; Iannuzzi et al., 1990a) and standardized (CSKBB, 1994).

The definition of a species-specific 'fragile site map' in domestic animals represents an important step toward a more precise characterization of the karyotype and an indication of the degree of chromosome stability/instability of the species. Such information can be highly useful for further studies on karyotype evolution, comparative genomics and mutational cytogenetics, particularly if the river buffalo species is considered as a 'bio-indicator' for environmental monitoring and nutritional security.

Material and methods

Cell cultures

Eight clinically healthy river buffaloes of the Italian Mediterranean breed were used for the investigation as follows: four (group A, two males and two females) reared on a farm located in the province of Naples and four (group B, two males and two females) reared in the province of Salerno. The choice to analyze subjects from two different farms/areas aimed at minimizing the genetic relationship between the investigated animals and the effects of environmental factors that could affect FS expression.

Conventional lymphocyte cultures were performed (De Grouchy and Roubin, 1965): after 48 h of culture, aphidicolin (APD) (Sigma, St. Louis, USA) was added to the cultures at a final concentration

of 0.15 µM as recommended by Rodriguez et al. (2002) and further incubated for 24 h. Six hours before the end of incubation, BrdU and H33258 (20 µg/ml each) (Sigma) were added to the cultures to label late replicating regions of the genome. For each animal a 'control' culture without aphidicolin addition was performed. The slides were subjected to RBA (Di Berardino and Iannuzzi, 1982) or RBG-band (Hayes et al., 1991) staining with acridine orange (0.01% in Sorensen buffer, pH = 7.0) or Giemsa and examined under fluorescent or bright field optics, respectively. Only metaphases with clear RBA- or RBG-banding and with at least one clear and unambiguous breakage were considered. A total of 400 breakages was scored in the aphidicolin-treated cultures, 50 per each animal, and localized on the standardized ideogram of the river buffalo (CSKBB, 1994).

Statistical analysis

ANOVA was performed to examine differences in the yield of breakages per chromosome among individuals and the Chi-square test was applied for detecting fragile sites. The Pearson correlation test was used to evaluate possible relationships between chromosome length and yield of FSs.

Results

Figure 1 shows an RBG-banded prometaphase plate of river buffalo with breakages on the X chromosome (Xq45) and on chromosomes 2q26, 7q33, 9q213 and 13q23; in Fig. 2 various breakages from RBG- (upper row) and RBA- (lower row) banded chromosomes, taken from partial and enlarged metaphases, are depicted.

Chromosomal distribution of breaks among individuals

Table 1 shows the chromosomal distribution of the breaks in the two groups of animals separated according to group (group A and B) and gender (males and females). No statistically significant difference was found in the chromosomal distribution of the breaks 'among' the eight animals investigated, as well as between the two groups A and B. However, when the animals were grouped according to gender, the differences between males and females were significant ($P < 0.01$), mainly

because of the sex chromosomes. The incidence of breaks on the sex chromosomes was 14% in the males (5 and 9% in the X and Y chromosomes, respectively), and 31% in the females, where the inactive X showed twice as many breaks compared to its active counterpart (21 vs. 10%, respectively).

Distribution of breaks 'among' river buffalo chromosomes

By assuming 15 as the mean value of breaks in the river buffalo haploid genome (400 breaks/26 haploid chromosomes), three classes (high, medium, low) of chromosomes could be established as follows: high frequency (30 breaks and above): the inactive X chromosome, chromosomes 9, 8 and the active X; medium frequency (16–29 breaks): chromosomes 1, 13, 7, 10, 2, 19, Y, 15, 17; low frequency (0–15 breaks): chromosomes 12, 4, 3, 5, 14, 16, 18, 22, 23, 20, 21, 6, 11, 24. No breaks were scored on chromosome arms 2p and 3p. Chi-square analysis revealed statistically significant differences ($P < 0.01$) among individual chromosomes, thus suggesting a 'non-random' distribution of the breaks. This finding was further confirmed by ANOVA ($P < 0.01$). When the yield of breaks per chromosome was correlated to the relative length of chromosomes, based on ten GTG-banded metaphases, the Pearson correlation test showed a positive value ($r = 0.41$; $P < 0.001$).

Chromosomal distribution of fragile sites

Based on the 438 bands of the standard RBG-banded karyotype, and assuming each band had an equal probability of breakage, the expected number of breaks per band for the 400 aberrations induced by APD was 0.91; $\chi 2$ analysis indicated that any band with three or more breakages was significantly damaged ($\chi 2 = 4.80$; d.f. = 1; $P < 0.05$) and therefore can be considered as a 'fragile site'. Out of 106 different breakpoints, 51 fragile sites were detected, which were distributed as follows: five fragile sites on chromosome 8; four on the active X chromosome, the inactive X chromosome, the Y chromosome and chromosome 7; three on chromosomes 1, 2, 10 and 13; two on chromosomes 3, 9, 12 and 22; one on chromosomes 5, 14, 15, 16, 17, 18, 19, 20, 21 and 23. No FSs were found on chromosomes 4, 6, 11 and 24.

Band localization of the fragile sites

Band localization of the FSs is reported in Table 2. The most fragile bands of the river buffalo karyotype were identified as follows: 9q213 with 24 breaks out of 400, 19q21 with 16; inactive Xq24 with 15; 15q23 and 17q21 with 13 and 13q23 with 12 breaks, respectively.

Discussion and conclusions

The chromosomal distribution of the APD-induced breakages did not vary significantly among the eight animals investigated. However, when the animals were grouped according to gender, the difference between males and females was statistically significant. In fact, in the males the yield of breaks on the X and Y chromosomes was 5 and 9%, respectively; in the females, the inactive X chromosome showed twice as many breaks compared to the active counterpart (21 vs. 10%, respectively). In other words, the females showed a higher rate of APD-induced breakages on sex chromosomes compared to the males (31 vs. 14%, respectively) and this difference was statistically significant ($P < 0.01$). This is in contrast with previous observations in pigs by Riggs et al. (1993) and in cattle by Rodriguez et al. (2002) who reported variations among animals due to an 'animal effect' rather than by gender.

In the present study, the yield of breakages per chromosome was found to be significantly different 'among' the chromosomes within the karyotype thus indicating a 'non-random' expression. In fact, some chromosomes such as the inactive X of the females, chromosomes 9, 8 and the active X chromosome of the males showed an over-representation of breakages, while others such as chromosomes 6, 11, 24 showed an under-representation. The low correlation index with the chromosome length ($r = 0.41$) suggests that other factors may be important in determining the final response. One of these factors might be the replication timing of the individual chromosome. This might suggest that chromosomes with higher yield of breakages could start replication later in S phase than the other chromosomes, thus resulting in a greater effect due to a longer aphidicolin exposure, which is known to act specifically in the late S-phase. Such explanation is supported by the fact that

156 in females the inactive (late replicating) X chromosome showed twice as many breakages compared
157 with its active counterpart. Specific investigations on the replication timing of individual
158 chromosomes might provide more insight into the complex phenomenon of FSs (Di Berardino et al.,
159 2002). Interestingly, there seems to be an absence of FSs on both BBU2p and BBU3p. These two
160 chromosome arms are homologous to BTA23 and BTA19, respectively. BBU2p (and BTA23)
161 contains the major histocompatibility complex (MHC) (Iannuzzi et al., 2003), while BBU3p (BTA19)
162 is a gene-rich chromosome that is almost completely euchromatic (Iannuzzi et al., 2003). Moreover,
163 these two chromosome arms are syntenic with HSA6p and HSA17, respectively. Interestingly, recent
164 data on FS expression in humans (Schwartz et al., 2006) show only two sites on chromosome 6
165 (6p25.1 and 6p22.2) and one on chromosome 17 (17q23.1) thus suggesting that some chromosomes
166 (or chromosomal regions) might be less prone to break than others, in relation to the presence/absence
167 of evolutionarily important genes such as e.g. the MHC gene complex.
168 Our data also revealed that the breakages were mainly located on the RBA or RBG negative bands
169 (heterochromatic bands) or at interband regions. Unlike the study reported by Rodriguez et al. (2002),
170 breakages were never observed on RBG positive bands. Such a discrepancy might be attributed to the
171 lower level of banding resolution used in that study: in fact, in more contracted chromosomes, a large
172 positive band may hide two or more sub-bands separated by negative bands where the breakage may
173 occur.
174 The fragile sites Xq21→Xq24 have been previously reported by Pires et al. (1998) in three river
175 buffaloes, two females and one male, but once again the lower level of resolution doesn't allow
176 comparison with our data.
177 By comparing the present results with those reported in cattle by Rodriguez et al. (2002), obtained
178 under the same experimental conditions, we observe a complete lack of homology in the location of
179 fragile sites between the two species. While in river buffalo the most fragile band has been found on
180 chromosome 9q213 with 24 breakages out of 400 (6%), followed by chromosome 19q21 with 16
181 (4%), chromosome 17q21 and inacXq24 with 15 (3.75%), in cattle the most fragile bands have been

found in totally different non-homologous chromosomes, such as 1q13 and Xq31, with 14 breakages out of 217 (6.45%), followed by chromosome 1q21 with 11 (5.0%), 3q22 and 5q32 with 9 (4.1%). It is difficult to explain the difference between these two species, which are known to share a great deal of chromosome banding (Di Berardino et al., 1981; Iannuzzi et al., 1990b; Gallagher and Womack, 1992; CSKBB, 1994) and gene homologies (Iannuzzi et al., 2003). One of the possible explanations might be seen in recent mutations in DNA composition and changes in DNA function which occurred after the five sets of centric fusions (autosomes) and sex chromosome rearrangements had taken place, thus differentiating river buffalo from the bovid ancestor (and cattle).

Preliminary gene mapping analysis of river buffalo reveals that the closest loci to some of the fragile sites detected in the present study contain genes such as RASA1 and CAST (9q214), NPR3 and C9 (19q19), OarCP09 (15q24), PLP and BTK (Xq24→q25) and EDNRB (13q22) (Iannuzzi et al., 2003), whose mutations are responsible for severe phenotypic malformations and immunodeficiency in humans and mice. The CAST gene has been considered a good candidate gene for determination of meat quality in pigs.

Recent data (Ruiz-Herrera et al., 2006) strongly support the view that at least some FSs are important hot spots for karyotype evolution, being the preferred sites for chromosome rearrangements. In the present instance, this may be true for the X chromosome evolution. Xq24, in fact, corresponds to one of the breakpoints related to the 'centromere transposition' or 'centromere repositioning' (with loss of constitutive heterochromatin) originating the submetacentric X chromosome of cattle from the acrocentric river buffalo one (Iannuzzi et al., 2000). In humans, fragile sites can be classified as rare and common, according to their expression frequency in the population. Within these two groups, subgroups can be set up according to their specific mode of induction in vitro (Sutherland and Hecht, 1985; Sutherland and Richards, 1995). A total of 31 rare fragile sites have been documented to date, and two (FRAXA and FRAXE) are associated with mental retardation, while for the others no proven phenotypic effects have been identified. Common fragile sites seem to play a key role in chromosomal rearrangements observed in malignant cells (for a review see Lukusa and Fryns, 2008). This seems

to be mainly due to the lack of functionality of important genes, associated with FSs that usually act as tumour suppressor (e.g. FHIT on FRA3B region, WWOX on FRA16D region). Furthermore, associations between common FSs and neuropsychiatric disorders have been postulated, especially between FRA6E and autosomal recessive juvenile Parkinsonism (Denison et al., 2003), and between FRA13A and idiopathic autism (Savelyeva et al., 2006). This may suggest that frequent recombination events at these sites could destabilize genes involved in the development and function of the central nervous system (Lukusa and Fryns, 2007). In this regard, farm animals could be used as 'in vivo' models to investigate these kinds of pathologies, especially cancer, to further clarify the role of fragile sites in cancer formation and development.

Further cytogenetic and molecular studies are needed to fully exploit the biological significance of the fragile sites and their relationships with animal biodiversity, environmental and nutritional security, and productively and reproductively efficient livestock.

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290 Fig. 1 RBG-banded prometaphase plate of river buffalo with breakages.

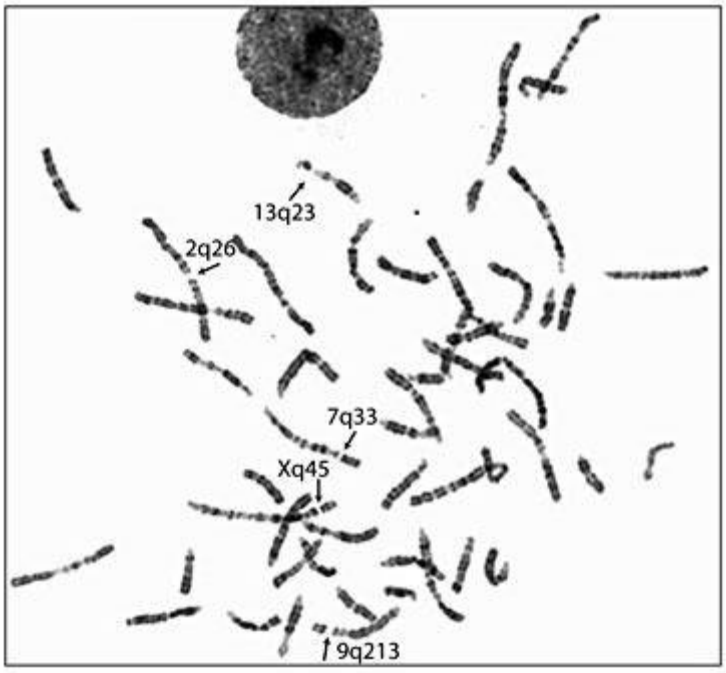

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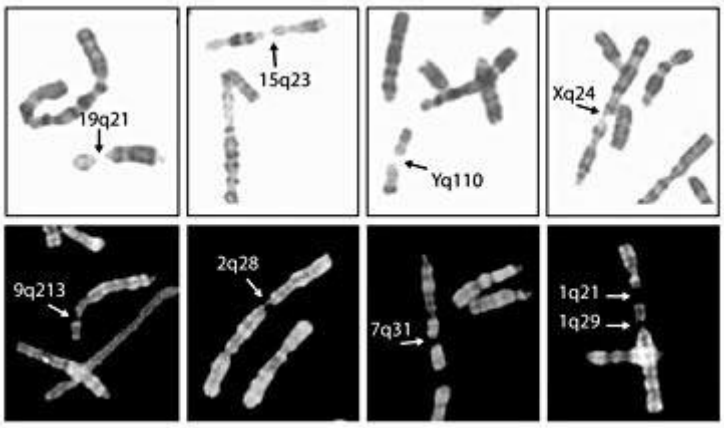
Fig. 2. Various breakages on RBG- (upper row) and RBA- (lower row) banded chromosomes from partial and enlarged metaphases.



297 Table 1. Breakages distribution (N in %) on individual chromosomes according to the farm (A or B)
298 and to gender

Chromosome	Group A N (%)	Group B N (%)	Males N (%)	Females N (%)	A + B N (%)
1	8 (4.0)	18 (9.0)	12 (6.0)	14 (7.0)	26 (6.5)
2	6 (3.0)	12 (6.0)	8 (4.0)	10 (5.0)	18 (4.5)
3	4 (2.0)	4 (2.0)	7 (3.5)	1 (0.5)	8 (2.0)
4	6 (3.0)	3 (1.5)	6 (3.0)	3 (1.5)	9 (2.2)
5	4 (2.0)	2 (1.0)	2 (1.0)	4 (2.0)	6 (1.5)
6	3 (1.5)	1 (0.5)	4 (2.0)	0	4 (1.0)
7	10 (5.0)	13 (6.5)	13 (6.5)	10 (5.0)	23 (5.7)
8	16 (8.0)	15 (7.5)	18 (9.0)	13 (6.5)	31 (7.7)
9	21 (10.5)	11 (5.5)	19 (9.5)	13 (6.5)	32 (8.0)
10	12 (6.0)	9 (4.5)	10 (5.0)	11 (5.5)	21 (5.2)
11	1 (0.5)	3 (1.5)	2 (1.0)	2 (1.0)	4 (1.0)
12	6 (3.0)	7 (3.5)	7 (3.5)	6 (3.0)	13 (3.2)
13	12 (6.0)	12 (6.0)	12 (6.0)	12 (6.0)	24 (6.0)
14	3 (1.5)	3 (1.5)	4 (2.0)	2 (1.0)	6 (1.5)
15	11 (5.5)	5 (2.5)	11 (5.5)	5 (2.5)	16 (4.0)
16	2 (1.0)	4 (2.0)	3 (1.5)	3 (1.5)	6 (1.5)
17	10 (5.0)	6 (3.0)	10 (5.0)	6 (3.0)	16 (4.0)
18	2 (1.0)	4 (2.0)	4 (2.0)	2 (1.0)	6 (1.5)
19	9 (4.5)	9 (4.5)	12 (6.0)	6 (3.0)	18 (4.5)
20	2 (1.0)	3 (1.5)	1 (0.5)	4 (2.0)	5 (1.2)
21	1 (0.5)	4 (2.0)	1 (0.5)	4 (2.0)	5 (1.2)
22	2 (1.0)	4 (2.0)	2 (1.0)	4 (2.0)	6 (1.5)
23	5 (2.5)	1 (0.5)	4 (2.0)	2 (1.0)	6 (1.5)
24	0	1 (0.5)	0	1 (0.5)	1 (0.2)
Autosomes	**156 (78.0)**	**154 (77.0)**	**172 (86.0)**	**138 (69.0)**	**310 (77.5)**
X	15 (7.5)	15 (7.5)	10 (5.0)	20 (10.0)	30 (7.5)
X-inactive	21 (10.5)	21 (10.5)	–	42 (21.0)	42 (10.5)
Y	8 (4.0)	10 (5.0)	18 (9.0)	–	18 (4.5)
Gonosomes	**44 (22.0)**	**46 (23.0)**	**28 (14.0)**	**62 (31.0)**	**90 (22.5)**
Total	**200**	**200**	**200**	**200**	**400**

299

300

Table 2. Identification of aphidicolin-induced fragile sites on RBA/RBG-banded prometaphase chromosomes of river buffalo

No. of breaks	Location
24[a]	9q213
16[a]	19q21
15[a]	17q21, inacXq24
13[a]	15q23
12[a]	13q23
11[a]	inacXq31
9[a]	8q16
8[a]	1q21, 10q12, inacXq22
7[a]	7q24, 8q31, Xq24, Xq31
6[a]	7q14, 7q31, 10q14, 13q21, 23q12, Xq22, inacXq13
5[a]	1q29, 2q13, 2q28, 12q21, 12q31, 13q12, Yq14, Yq110
4[b]	8q21, 8q33, 9q21, 10q21, 14q12, 16q14, 18q21, Xq13, Yq12, Yq16
3[c]	1q44, 2q26, 3q14, 3q16, 5q15, 7q22, 8q14, 20q12, 21q21, 22q13, 22q21

[a] $P < 0.001$; [b] $P < 0.005$; [c] $P < 0.05$.