ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 33 (2005) 1209–1214

Age-associated skewing of X-inactivation ratios of blood cells in normal females: a candidate-gene analysis approach

Pierre Chagnon[a,b], Sylvie Provost[a], Claude Belisle[a], Véronique Bolduc[a], Marianne Gingras[a], and Lambert Busque[a,c]

[a]*Research Center, Maisonneuve-Rosemont Hospital, University of Montreal, Montreal, Quebec, Canada;*
[b]*Institute for Research in Immunology and Cancer, University of Montreal, Montreal, Quebec, Canada;*
[c]*Department of Hematology, Maisonneuve-Rosemont Hospital, University of Montreal, Montreal, Quebec, Canada*

(Received 10 April 2005; revised 17 May 2005; accepted 17 June 2005)

X-inactivation is a random process that occurs in females early during embryogenesis. Females are mosaics with an equal proportion of cells with the paternal (Xp) or maternal X-chromosome (Xm) in the active state. However, close to 40% of healthy females aged more than 60 y.o. present a significant skewing of X-inactivation ratios (Xp:Xm >3 :1). The exact etiology of this age-associated skewing (AAS) in blood cells is unknown. We hypothesized that AAS is due to hemizygous cell selection caused by allelic variants in hematopoiesis or cell survival genes. To test this hypothesis, we recruited 700 unrelated healthy females of French Canadian ancestry aged more than 60. We determined X-inactivation ratio at the HUMARA locus. We genotyped 81 different SNPs, using TaqMan® technology, in 15 different candidate genes with known role in hematopoiesis, cell cycle, or X-inactivation. Extensive statistical analyses were conducted and demonstrated that none of the 15 candidate genes investigated contribute significantly to AAS.

In the present investigation, 81 single nucleotide polymorphisms (SNPs) in 15 X-linked candidate genes were screened for association with the age-associated skewing (AAS) of X-chromosome inactivation ratios that occur in blood cells of normal elderly women. AAS was analyzed as an independent quantitative trait on a cohort of 700 women from a French-Canadian founder population.

X-chromosome inactivation allows transcriptional silencing of one of the two Xs in every female somatic cell to equalize X-linked gene dosage between XX females and XY males. This phenomenon is random, occurs early during embryogenesis, is stable over time, and is maintained in the progeny of every cell. Therefore, females are mosaics harboring equal proportions of cells with the paternal (Xp) and maternal (Xm) X chromosomes in the active state (Xp/Xm = 1:1). Blood cells, however, present an increased incidence of skewing of X inactivation ratio (Xp/Xm > 3:1 or < 1:3) over time, which can reach up to 40% in females aged older than 60 years [1,2].

Offprint requests to: Lambert Busque, M.D., F.R.C.P.C., Centre de Recherche, Hôpital Maisonneuve-Rosemont, 5415 Boul. de l'Assomption, Montréal, Québec, Canada, H1T 2M4; E-mail: lbusque.hmr@ssss.gouv.qc.ca

AAS is an intriguing phenomenon of unknown etiology. Stochastic causes, such as clonal hematopoiesis or stem cell depletion with secondary clonal dominance, have been hypothesized. However, several recent observations have suggested an X-linked genetic basis to AAS, including age-associated skewing in safari cats, which always occurs in favor of the same X chromosome [3], and concordance in incidence and direction of skewing in three twin studies [4–6].

We have hypothesized that the AAS phenomenon observed in blood cells is caused, at least in part, by allelic variants in gene(s) involved in hematopoiesis and located on the X chromosome. In females, heterozygous for such genes, a beneficial allele could confer a selective advantage to cells in which it is active. Gene(s) responsible for AAS may have a significant role in stem cell kinetics [3], and their identification may lead to a better understanding of the biology of aging hematopoiesis.

In order to test this hypothesis, we have chosen to use a candidate-gene approach, as there are several genes on the X chromosome that have a demonstrated role in hematopoiesis or cell division, and because association studies are potentially a more powerful approach than traditional linkage studies for identifying causal genes in complex traits [7]. Fifteen different genes located on the X chromosome

0301-472X/05 $–see front matter.
doi: 10.1016/j.exphem.2005.06.023

were selected for their potential involvement in AAS because of their roles in X-inactivation mechanisms (XIST), hematologic disorder (PIGA for paroxysmal nocturnal hemoglobinuria), selection and proliferation of hematopoietic stem cells (GATA1, CDX4, MLLT7), differentiation of white blood cells (IL2RG), or for their association with skewing observed in female carriers of genes for pathological conditions like Wiskott-Aldrich syndrome (WAS) [8]. This approach is not comprehensive, as numerous other genes are potential candidates. There are, on the X chromosome, more than 25 kinase family genes, 9 other interleukin-related genes, and dozens of genes implicated in cells proliferation. However, the selected genes have either been implicated in hematologic disorders or have been more extensively characterized, and constitute a strong starting point for such a candidate-gene strategy.

Seven-hundred unrelated healthy females of French-Canadian ancestry were recruited. All procedures were undertaken with approval from the Maisonneuve-Rosemont Hospital's Ethics Committee and with the subjects' written informed consent. All subjects met the following criteria: 55 years of age or older and no blood diseases or active cancer. Mean age of the individuals was 71.1 years old, with a standard deviation of 9.7 years (range 55–100 years). Following criteria formulated by Zondervan and Cardon [9], we have evaluated that our cohort has a statistical power of 95% to detect DNA variants that will increase by twice the probability to develop the phenotype, if the causal allele frequency is above 0.2. However, if the causal variants increase the allelic odds ratios by only 1.3, chances to detect such variants decrease. The size of our cohort is thus largely sufficient to detect major contributors of AAS, but probably not minor contributors.

Peripheral blood samples were obtained from each subject. Complete blood count and white cell differential were performed. Two cell populations were isolated from all samples: polymorphonuclear cells (PMN) using standard density gradient centrifugation and T lymphocytes using a fluorescence-activated cell sorter or magnetic beads.

Based on a highly polymorphic CAG repeat in the first exon of the human androgen receptor gene located on the X chromosome, a DNA methylation-sensitive assay (HUMARA) was used to distinguish the inactive X chromosome from the active one in PMN and T cells [10]. Deviation from random X-inactivation ratio was measured by the degree of skewing score (DS). Values ranged from 0 (random X inactivation) to 0.5 (maximal skewing) [11]. Duplicate assays were performed on each sample. Correlation with transcription-based reverse transcriptase polymerase chain reaction assays at the IDS and G6PD loci was also used to further validate the HUMARA assay (data not shown). Mean value of DS was 0.25 (SD = 0.14) and 0.17 (SD = 0.12) for PMN and T cells, respectively. PMN are thus more subject to skewing in elderly women compared with T cells.

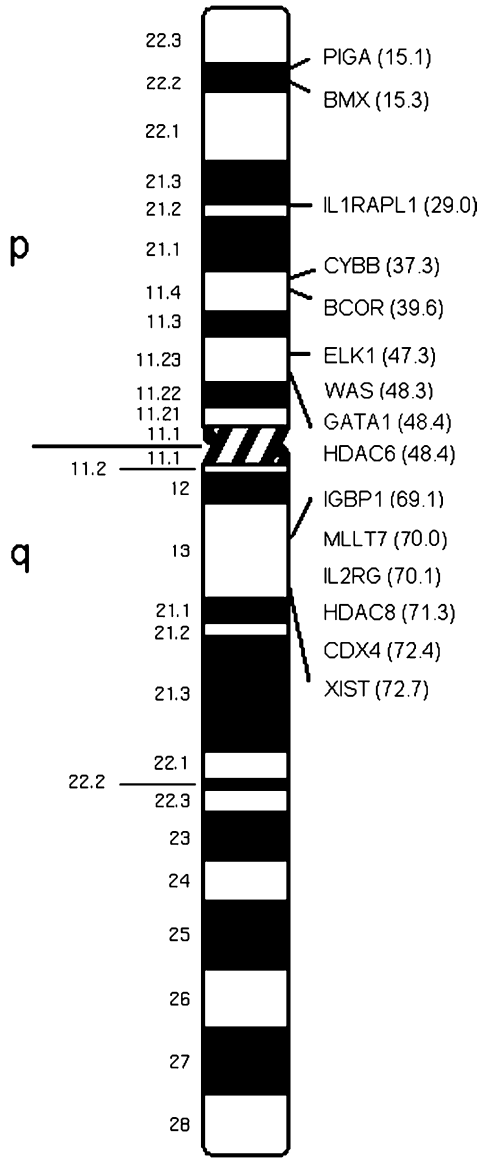

Figure 1. Map of the X chromosome indicating the position (in megabase) of the genes studied.

Fifteen candidate genes located on the X chromosome (see chromosomal position in Fig. 1) were selected and analyzed: phosphatidylinositol glycan, class A (PIGA, Xp22.1); BMX nonreceptor tyrosine kinase (BMX, Xp22.2); interleukin-1 receptor accessory protein-like 1 (IL1RAPL1, Xp22.1-p21.3); cytochrome b-245, beta polypeptide (CYBB, Xp21.1); BCL6 co-repressor (BCOR, Xp11.4); ELK1, a member of the ETS oncogene family (ELK1, Xp11.2); Wiskott-Aldrich syndrome (WAS, Xp11.3); globin transcription factor 1 (GATA1, Xp11.23); histone deacetylase 6 (HDAC6, Xp11.23); immunoglobulin CD79A binding protein 1 (IGBP1, Xq13); myeloid/lymphoid or mixed-lineage leukemia translocated to 7 (MLLT7, Xq13.1); interleukin-2 receptor, gamma (IL2Rg, Xq13.1); Histone deacetylase 8 (HDAC8, Xq13);

Table 1. List of single nucleotide polymorphisms analyzed in our cohort

	Genes	dbSNP_ID	X-chromosome position	MAF	PMN (p value)	T cell (p value)
1	PIGA	rs3434	15 097 271	0.431	.35	.53
2		rs3087965	15 097 539	0.423	.56	.14
3		rs3661	15 097 659	0.421	.79	.28
4		rs5935953	15 100 327	0.422	.55	.12
5		rs5935954	15 100 881	0.393	75	.33
6		rs5978726	15 101 317	0.478	.64	.21
7		rs5935955	15 105 337	0.423	.65	.14
8		rs2543	15 105 501	0.454	.34	.25
9	BMX	rs979848	15 288 025	0.392	.26	.78
10		rs1399202	15 302 193	0.212	.83	.26
11		rs963447	15 313 153	0.381	.15	.58
12		rs233576	15 321 158	0.357	.16	.86
13		rs233569	15 324 411	0.352	.21	.88
14		rs2873356	15 333 425	0.351	.42	.97
15	IL1RAPL1	rs1419851	29 701 387	0.138	.39	.78
16		rs5973335	29 704 159	0.449	.98	.71
17		rs17885035	29 727 832	0.042	.79	.67
18	CYBB	rs4422908	37 397 743	0.172	.79	.59
19		rs4827298	37 407 876	0.232	.33	.17
20		rs5964125	37 414 668	0.157	.52	.38
21		rs5963309	37 415 269	0.158	.56	.36
22		rs5964149	37 425 911	0.159	.51	.38
23		rs5964151	37 426 946	0.161	.49	.54
24	BCOR	rs5963725	39 667 880	0.215	.92	.65
25			39 669 880	0.048	.62	.31
26		rs5963731	39 679 686	0.109	.58	.19
27		rs12014902	39 683 166	0.102	.98	.44
28		rs6520618	39 689 130	0.122	.57	.11
29		rs5917933	39 689 563	0.138	.44	.86
30	ELK1	rs1048118	47 240 054	0.239	.87	.07
31		rs2765814	47 249 469	0.281	.21	.37
32		rs2765815	47 249 988	0.276	.38	.49
33		rs1059579	47 254 654	0.004	.76	.94
34		rs1998837	47 259 119	0.323	.06	.63
35		rs2742917	47 260 153	0.291	.09	.86
36		rs2283735	47 263 995	0.289	.21	.73
37	WAS	rs235423	48 300 900	0.058	.11	.93
38		rs2737799	48 303 362	0.011	.17	.08
39		rs2737800	48 306 157	0.039	.19	.71
40	GATA1	rs1554987	48 377 762	0.167	.63	.21
41			48 401 140	0.081	.91	.46
42		rs5906709	48 403 154	0.162	.88	.82
43	HDAC6	rs2008290	48 424 765	0.166	.28	.86
44		rs2075840	48 429 037	0.171	.79	.93
45		rs2075837	48 433 087	0.171	.79	.89
46		rs1985411	48 434 345	0.172	.79	.82
47	IGBP1		69 153 350	0.104	.58	.14
48		rs606039	69 153 405	0.111	.97	.86
49		rs5936856	69 168 496	0.148	.41	.91
50	MLLT7	rs12013673	70 100 119	0.428	.21	.93
51		rs5980742	70 104 651	0.435	.11	.55
52	IL2RG	rs11574627	70 110 221	0.016	.38	.75
53		rs11574625	70 113 617	0.101	.86	.28
54	HDAC8	rs660392	71 332 533	0.143	.86	.63
55		rs576502	71 344 394	0.137	.62	.45
56		rs11093377	71 374 933	0.022	.09	.03
57	CDX4	rs2273781	72 087 212	0.135	.86	.85
58		rs2189413	72 142 853	0.127	.61	.19
59		rs2493426	72 361 062	0.082	.71	.51
60			72 449 665	0.262	.31	.13

(*continued*)

Table 1 (*continued*)

	Genes	dbSNP_ID	X-chromosome position	MAF	PMN (p value)	T cell (p value)
61			72 450 002	0.051	.62	.81
62		rs1554917	72 455 183	0.332	.26	.11
63		rs2812027	72 458 216	0.147	.60	.97
64		rs4892781	72 458 659	0.007	.37	.24
65		rs16992386	72 459 546	0.012	.84	.11
66	XIST	rs1337657	72 784 062	0.128	.78	.16
67		rs798624	72 786 269	0.061	.82	.25
68		rs798615	72 796 668	0.073	.48	.88
69		rs1620574	72 826 838	0.313	.96	.44
70		rs1794213	72 828 761	0.093	.12	.64
71		rs1009948	72 836 694	0.064	.83	.98
72		rs1894271	72 849 460	0.136	.76	.79
73		rs6527	72 849 912	0.076	.99	.99
74		rs6528	72 851 594	0.022	.36	.99
75		Familial mutation	72 855 613	0.0015	.73	.94
76		rs530957	72 864 882	0.047	.61	.68
77		rs575648	72 870 288	0.155	.61	.21
78		rs542191	72 870 408	0.134	.73	.038
79		rs195677	72 874 806	0.081	.62	.07
80		rs195679	72 880 399	0.077	.85	.79
81		rs1243760	72 909 754	0.315	.83	.18

Positions of single nucleotide polymorphisms (SNPs) are based on National Center for Biotechnology Information (NCBI) human genome assembly build 35 (2005). p Value of statistical analysis (analysis of variance) is also indicated for polymorphonuclear cells (PMN) and T cells. SNPs that have no NCBI single nucleotide polymorphism database identification (dbSNP ID) were identified in our cohort by sequencing the coding regions of some candidate genes in 24 individuals. MAF = minor allele frequency.

caudal-type homeo box transcription factor 4 (CDX4, Xq13.2); and X inactive-specific transcript (XIST, Xq13.2).

Using TaqMan technology on an ABI PRISM 7000, we genotyped 81 different SNPs in our population. SNPs were selected from The SNP consortium (http://snp.cshl.org/), the National Center for Biotechnology Information's single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/) and the University of California Santa Cruz Genome Bioinformatics site (http://genome.ucsc.edu). SNPs were chosen on the basis of their heterozygosity (when available), their position within the candidate genes, and linkage pattern (Table 1). In order to maximize the likelihood of finding a potential association, several SNPs were chosen for each gene (average of 1 SNP per 3.5 kb of genomic DNA sequence, except for HDAC8 and IL1-RAPL1 that are larger genes).

Quantitative statistical analysis was performed for each SNP by an analysis of variance (ANOVA) using the three possible genotypes and the DS score as a quantitative trait. Moreover, ANOVA was used to determine whether heterozygote individuals have a different level of DS compared to homozygotes. Our data show that none of the 81 SNPs analyzed presents a significant difference in DS scores for the different genotypes. In fact, the smallest p value observed was for a SNP located in the XIST gene (rs542191) associated with skewing in T lymphocytes (p = 0.035) (Table 1). Individuals heterozygous for this marker present a slight increase in DS (0.177) compared to the homozygous individuals (DS = 0.154). However, with the Bonferroni correction, significance was lost. In addition, no significant results were observed for this marker when using skewing in PMN cells as the phenotype. Thus, this slight association is probably only an artefact. We also compared the genotype frequencies between the groups of skewed women (PMN or T cells) by qualitative assignation of the trait, i.e., individuals having a DS score above 0.25 were considered skewed and individuals having a DS score below 0.15 were considered nonskewed. Chi-square analysis showed no significant results, including the XIST polymorphism (data not shown).

We also analyzed a XIST promoter mutation (SNP no. 75, Table 1) [12], previously identified in two unrelated families showing preferential inactivation of the mutated X chromosome. Three women heterozygote for that mutation were found in our cohort. They present a level of skewing comparable to the noncarrier women (DS in PMN = 0.22 and DS in T cells = 0.17). Thus, this mutation is present in the French-Canadian population and is not associated with an excessive skewing, as described by Plenge et al. [12].

Pairwise linkage disequilibrium, calculated for each gene with GOLD software [13], revealed a strong linkage disequilibrium pattern between most SNPs of a specific gene (Fig. 2). This observation demonstrates that for most genes only a few common haplotypes are present in the French-Canadian population, and consequently, the number of SNPs required to analyze a given gene is small.

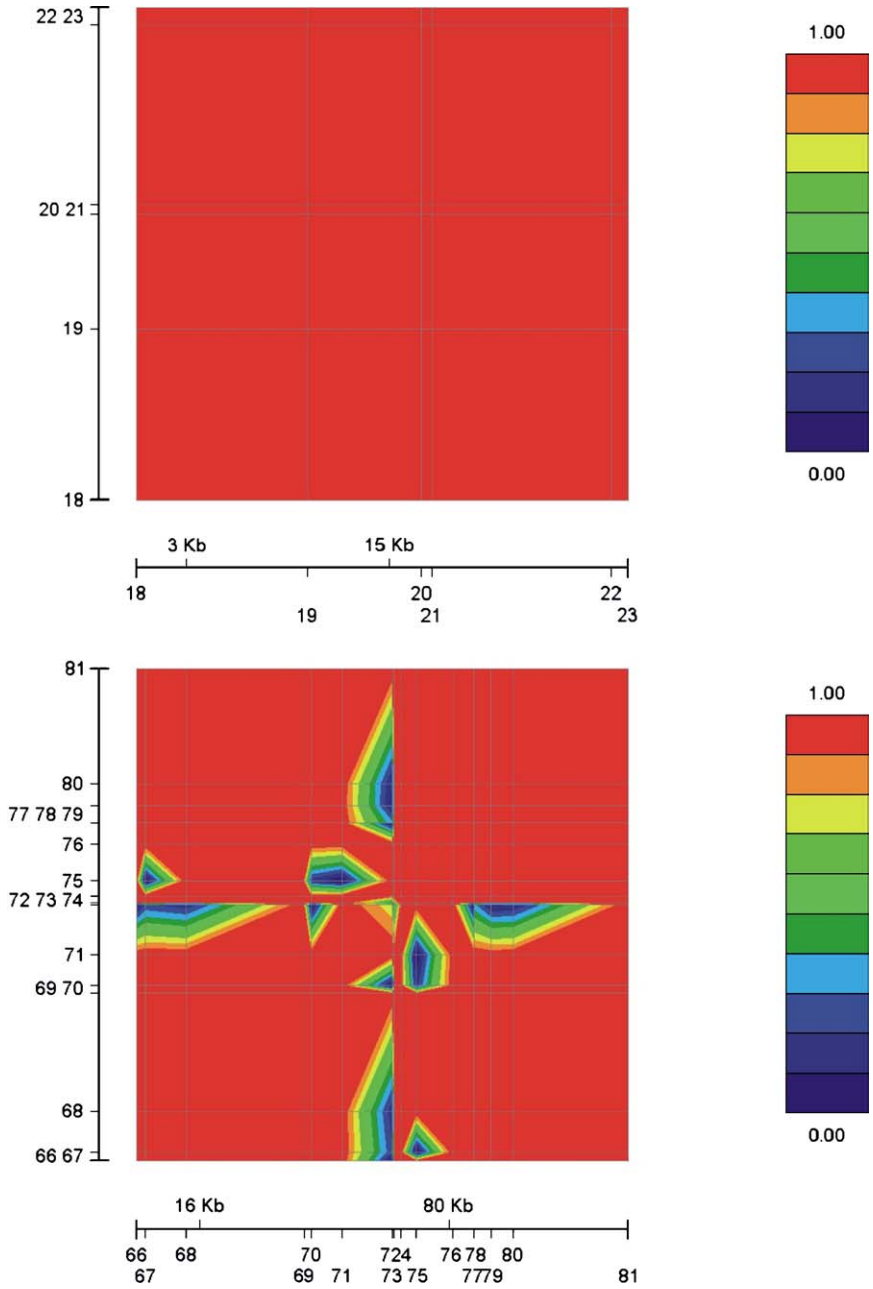

Figure 2. Pairwise linkage disequilibrium pattern in the French-Canadian population for some candidate genes of our study (CYBB: upper panel; XIST: lower panel). The graph was generated by GOLD (Abecasis and Cookson, 2000). Single nucleotide polymorphism numbers (18 to 23 = CYBB; 66 to 81 = XIST) correspond to the position in Table 1. Red color indicates a complete linkage between two polymorphisms, whereas blue color indicates no linkage.

For example, Figure 2 shows that the pattern of linkage disequilibrium (LD) for the CYBB gene is complete, even for SNPs separated by 30 kb genomic DNA sequence. All other genes with a similar length demonstrated complete or almost complete LD pattern. Over a longer distance, such as the XIST region that was investigated with 16 SNPs, we observed complete and partial LD. This illustrates that in the French-Canadian founder population, LD can extend to over 100 kb.

Finally, we applied a statistical algorithm for reconstructing haplotype phases of each gene. Permutation tests were performed in association analyses using UNPHASED software package (QTPHASE) [14]. All analyses yielded negative results, confirming the ANOVA test results.

Considering that this study was performed on a founder population, that the haplotype blocks are generally large in such populations, and that the size of the studied genes was relatively small, we can conclude that none of the candidate genes selected for analysis in this study is a major genetic determinant of the AAS phenomenon. We have therefore ruled out the involvement of very important hematopoietic

genes such as PIGA, WAS, CDX4, and GATA-1, which were prime candidates for the hemizygous cell selection theory. This indicates that other genes on the X chromosome are responsible for the AAS phenomenon. It is also possible that the AAS is a more complex trait, caused by several different genes with minor contributions. If this is the case, the contribution of each gene to the trait may vary between individuals, depending on the relative selective effects of the alleles, and an extremely large cohort would be needed to identify the different causative genes.

Because of the importance of identifying the causative gene for AAS, other approaches, such as a complete linkage analysis using sib-pair individuals or an association study that would map the entire X chromosome at a very high density, should be considered.

Acknowledgments

The authors are grateful to the individuals who have participated in this study for generously donating samples. This study was supported in part by HemaX Genome. We also thank Dr. Anik Boudreau for helpful comments and correction of the manuscript.

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