Research Article

Low-Level Gonosomal Mosaic (LLGM) In Female Reproduction

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Abstract

Background: LLGM is the most prevalent chromosomal aberration in the population of females with reproductive impairment. Its significance is still elusive. The aim of the study was to assess the significance of LLGM in female with reproductive impairment (tested group) and review the published data. **Method**: Cytogenetic and fluorescent in situ hybridization (FISH) analyses on cultured and uncultured blood samples. **Results**: There was no significant difference in the incidence of LLGM in patients from the tested group vs. control. The mean of X aneuploidy cells proportion was 2.2 % in the tested vs 1.2 % in control group by cytogenetic examination (p> 0.05). The frequency of X aneuploidy cells in tested group vs. control was significantly higher when the examination was performed by FISH: mean = 5,4 % in cultured and 6,4 % in uncultured samples vs. 3.9 % and 3.6 % resp. in the control (*p <0.05).* **Conclusion**: FISH provides more detailed assessment of X aneuploidy cell proportion in patients treated for infertility. Incidence of LLGM and frequency of X aneuploidy cells increases with age in females generally. Accelerated increase of X aneuploidy occurrence observed in the infertile group implies that the mitotic error occurs more frequently in females with reproductive failure. IVF outcomes did not differ between tested and control cohort. Our results show that the patients with LLGM show higher predisposition to recurrent miscarriage (P<0.05).

Keywords: infertility, low-level gonosomal mosaic, FISH, recurrent spontaneous abortions (RSA)

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Introduction

Fertility declines significantly in recent years according to Rostand et al. (2013). Basic research helps to define genes and mechanisms responsible for development, function and regulation of the human reproductive system. However, a considerable amount of infertile couples are assigned to the category of idiopathic infertility, which is often bypassed with assisted reproductive technologies (ART) at the expense of high costs and accompanied by safety or ethical concerns. One of the most common causes of infertility or recurrent spontaneous abortions (RSA) is the genetical reason from which the chromosomal abnormalities take an important place. Among them the low-level gonosomal mosaicism (LLGM) (minority mosaic of hypo- or hyperdiploid X chromosome) shows a high incidence in females with reproductive impairment as it was published in papers written by Horsman et al. (1987), Gekas et al. (2001), Morel et al. (2004), Montag et al. (1997), Meschede et al. (1998), Mau et al. (1997), Čapkova et al. ((2004), Gekas et al. (2001), Scholtes et al. (1998), Sonntag et al. (2001), Peschka et al. (1999). Its significance has not been fully explained firstly due to the heterogeneity of LLGM definition used by various authors, secondly because the X aneuploidy shows a strong correlation with the age of females which means that a different age group has a different risk of reproductive problems as was published previously by Ford and Russell (1985), Guttenbach et al. (1995), Nowinski et al. (1990), Russell et al. (2007). Finally, different methodologies described in various studies make the final comparison of the results difficult. Toncheva et al. (1994) mentioned that clarifying the phenotypic relationships of chromosomal mosaicism is an important issue in cytogenetic diagnosis and genetic counselling. Studies performed by Sonntag et al. (2001) and Voight et al. (2004) confirmed that the LLGM does not show any impact on the outcome of IVF. Despite this fact, it has so far been unclear why the frequency of X- aneuploidy cells is so high in the population of females with reproductive failure. Ploidy of the X chromosome in the lymphocytes is mostly studied by the conventional cytogenetic method. However, this method is not optimal: the number of evaluated lymphocytes is limited, and only dividing cells are included in the assessment. The molecular-cytogenetic method (FISH) provides us with the possibility to detect gonosomal constitution also in interphase nuclei of uncultured lymphocytes, and it is the most appropriate method of confirming suspected mosaicism. This technique can also eliminate problems of the presence of culture artefacts. A similar method was used previously in the study of Patton et al. (2010). The aim of the study was to compare the incidence of the LLGM (and X aneuploidy cell proportion) in a group of female patients treated for infertility and the control cohort by the conventional method and by fluorescent in situ hybridization (FISH) in cultured and uncultured samples of peripheral blood. Correlation with age of females and relevant clinical data are included.

Patients and Methods

Cytogenetic Study

Routine cytogenetic analysis and FISH analysis were done in the group of 70 females, who were scheduled for IVF treatment in the Centre of Assisted Reproduction (median age = 30; range 19 – 42).The control group consisted of 83 females with at least two spontaneous conceptions without miscarriage history (median age = 32,5; range 22 – 46). The females were recruited mostly from the patients of the Department of Clinical Genetics and Foetal Medicine, where the blood sampling was taken as part of biochemical screening of pregnant females. All patients signed the informed consent. Confirmed fertility was defined as at least one natural pregnancy, no history of recurrent miscarriages, or any fertility treatments.

Cultured preparations were processed using standard cytogenetic procedures and evaluation and number scored metaphases

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respected "ECA Cytogenetic guidelines". 15 - 20 metaphases were analyzed; the number of analyzed metaphases was increased at min 30 cells - appropriate confidence limits using Hook´s tables (1977) for lymphocyte cultures- when single cell aneuploidy was detected in the sample. Gonosomal mosaicism was defined as low-level (LLGM) when at least two or more of the analyzed cells but less than 20 % displayed the constitution 45,X or 47,XXX or X polysomy.

Every single cell aneuploidy was counted irrespective of whether the sample fulfilled the criteria of mosaicism to assess the frequency of X aneuploidy *(45,X, 47,XXX, X polysomy)* cells in both cohorts.

FISH study

For FISH we used X specific centromere probes (DXZ1) from Qbiogene (Heidelberg, Germany). Procedures were carried out according to the manufacturer´s recommendation. FISH was successfully performed on cultured lymphocytes in the test group of 66 females and 51 females of the control group. Interphase FISH was successfully performed on cultured lymphocytes in the test group of 54 females and 46 females of the control group. Uncultured blood was treated directly with hypotonic solution, 0.075 mol/l KCL, before fixing in methanol: acetic acid (3 : 1). At least 100 cells were scored in FISH tests (mean=170).

Clinical Data

The data of the control cohort were used for calculation of the "cutoff value" in FISH method. The cutoff (value discriminating between control and pathological level) was calculated separately for cultured (7.78 %) and uncultured blood (6.81 %) respectively as the mean plus 1.96 SD as was described previously by Kuo and Guo (2004). The cohort of 110 infertile females treated by ICSI in the Centre of Assisted Reproduction was divided into three groups according to the detection of LLGM without detected mosaic - control (N=33)

or with mosaic either proved by standard cytogenetic method (N=44) or FISH with the cutoff value (N=33). The following parameters were assessed: previous pregnancies; previous spontaneous abortions; recurrent spontaneous abortions; ICSI cycles/patient; number of oocytes retrieved; fertilisation rate.

Statistics

Results between the test and control group were tested for significance using the Mann-Whitney U-test and the chi-square test as appropriate. A p-value of $p < 0.05$ was considered to show a significant difference.

Results

The incidence of LLGM (at least two cells in sample show X aneuploidy) in the group of 70 patients was 14 (20 %) vs.11 (13.58 %) in the control group of 83 healthy females (p>0.05). X chromosome loss was detected in 10 %; X chromosome gain in 2.85 % and presence of the both cell lines in 7.15 % of patients vs. 8.64 %females with X loss and 4.94 % females with both of the cell lines in the control.

The mean of proportion of X aneuploidy cells was 2.2 % (SD=3,743; R 0-19) in the tested group vs 1.2 % (SD 2,37; R 0-16) in the control group by cytogenetic examination. Although the frequency of aberrant cells was higher in the group of infertile females, a significant difference was not confirmed (*p >0.05*). The mean of proportion of X aneuploidy cells was 5,4 % (SD=2.945; R 1,7-20) in the infertile group vs. 3.9 % (SD=1.98; RI 0.8-10) in the control group of cultured samples tested by FISH (p<0.05). The mean of proportion of X aneuploidy cells was 6,4 % (SD=2.86; R 1.7-13.5) in the infertile group vs. 3.6 % (SD=1.64; R 0-7.6) in the control group of uncultured samples tested by FISH (p<0.05).The frequency of X aneuploidy cells was significantly higher in the infertile group than in the control group for cultured and uncultured samples tested by FISH (tab. 1, fig. 1).

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The frequency of aberrant cells similarly as the incidence of LLGM in females increases with age (fig. 2). The differences between particular age groups were not significant.

Analysis of clinical data shows a significant difference between the incidence of RSA in the patients without LLGM (0 %) and those who have LLGM detected with FISH 4 (12,12 %)(P<0.05). In the LLGM group is the highest occurrence of females older than 35 (tab. 2).

Discussion

The range of detected LLGM in the group of female patients with reproductive failure varies from 2,77 % to 16 % in so far published studies (see tab. 3a). LLGM is the predominant chromosomal aberration in this group of females. However, it is also detected in females with normal reproductive capacity as it was published previously by Horsman et al. (1987). There was a significant difference in the frequency of X aneuploidy cells in the group of females treated for infertility vs. control group by FISH in cultured and uncultured samples in our study. The cutoff values for cultured and uncultured cells were 7.78 % and 6.81 % resp. The frequency of sex aneuploidy from 6 % showed significant relevance in the study of Homer et al. (2010).

The majority of studies - Peschka et al. (1999), Nowinski et al. ((1990), Voight et al. (2004) - are based on cytogenetic analysis and clinical data are related to the incidence of LLGM (defined as at least two aberrant cells in the sample) in female. Voight et al. (2004), Sonntag et al. (2001) and Meschede et al. (1998) used for determining of the significance of the low-level gonosomal aneuploidy 15, 10 and 6 % respectively cutoff for LLGM. These results are biased by number of checked cells, culture artefacts and different cutoff values used by these authors. Moreover, the age of females was not taken into account. Scoring of all X aneuploidy cells irrespective of LLGM definition was published by Ford and Russell (1985),

Nowinski et al. (1990) and Kuo and Guo (2004) in studies of LLGM and age correlation (sees tab. 3b). FISH has not been used by many authors previously, and comparison to the interval of referral values is essential. Patton et al. (2010) detected the frequency of aberrant cells in the group of fertile females lower than 5 % by FISH. Similar results were obtained in our study in the control cohort.

The correlation between infertility and LLGM has not yet been confirmed. Sonntag et al. (2001) and Voight et al. (2004) proved that this chromosomal aberration is without any influence on the outcome of IVF in females treated for infertility. However, the results of studies written by Homer et al. (2010), Kuo and Guo (2004) and Voight et al. (2004) imply that the patients with LLGM show higher predisposition to spontaneous abortions. Our data lead to the same conclusion (frequency of RSA 12,12 % with LLGM vs 0 % without LLGM). Magli et al. (2002) suggests that the constitutional carriers of sex chromosomal mosaicism are predisposed to autosomal mosaicism in the embryo due to the probable inborn mitotic error of cell division which has a higher tendency in the female than in the male. Kovaleva et al. (2005) published two mechanisms that contribute to women´s susceptibility to minor mosaicism: sex-specific cell loss and sex-specific centromere instability. The occurrence of LLGM is age dependent. Fitzgerald (1983) described that the X hypoploidy (strongly associated with age) occurs mostly as a result of premature centromere division. Our clinical data imply that higher frequency of X aneuploidy cells is mostly influenced by the age of the patient. There were significantly more patients aged above 35 years in the groups with LLGM (proved either cytogenetically or FISH) compared to the group without LLGM in the clinical data assessment. The frequency of X aneuploidy cells was increased in age group above 35 years in comparison to younger (below 35) one but not significantly. Guttenbach et al. (1995) found a significant correlation only in women aged above 51. The frequency of X aneuploidy cells in particular age groups in

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the tested cohort resembles the frequency of X aneuploidy cells in fertile females of the older age group. This finding suggests that in females with LLGM errors of cell division occur more often than in females with normal reproductive capacity. If the malsegregation of chromosomes is associated with aging than we might speculate that the chronological age of females treated for infertility might differ from biological one and then these patients are more prone to acceleration of cell senescence. A similar idea was presented in the studies of Magli et al. (2002) and Nowinski et al. (1990). This hypothesis is also supported by the fact that there is a relatively high proportion of single cell aneuploidy of different chromosomes in females treated for infertility that was previously published by Peschka et al. (1999). Leach et al. confirmed by study of telomere length that acquired somatic cell aneuploidy is associated with aging. The association of age and infertility and age and pregnancy loss were confirmed by Grande et al. (2012). The authors of so far published studies have not been yet consistent in the correlation between LLGM and pregnancy loss.

The highest frequency of RSA was detected in the group of females treated for infertility with the X aneuploidy cells proportion higher than the cutoff. Despite that the mean age (31) in this group did not significantly differ from the other subgroups there was the highest rate of women aged above 35 (21 %)(tab. 2). It might suggest that females with LLGM are prone to recurrent abortions, and the LLGM could be a marker for biological age of patients. In a study by Devi et al. (1998) the higher incidence of LLGM is in patients with idiopathic premature ovarian failure. Kuo and Guo (2004) also imply the possible presence of diminished ovarian reserve in the presence of LLGM, which is associated with the spontaneous abortions or a higher rate of aneuploidy in foetuses. On the contrary according to study of Homer et al. (2010) the sex chromosome mosaicism below 30 % has no impact on the ovarian reserve of females. However, his study confirmed significantly higher rate of RSA in the group of patient with LLGM below 30 %.

Conclusion

The significant difference in frequency of X aneuploidy cells was confirmed between tested and control cohort by FISH. However, outcome of IVF was not significantly different in patients with LLGM from the patients without this chromosomal aberration. Females with LLGM detected by FISH showed significantly higher tendency to RSA, which appears to be influenced by age since there were significantly more patients older than 35 years in the group with LLGM detected by FISH than in the control. By using FISH, a large number of cells can be rapidly counted which facilitates further assessment of low-level aneuploidy. The calculation cutoff value for different methods, tissues and age group should be used. Detection of X aneuploidy proportion higher than the cutoff in patients should not be underestimated due to the possible presence of gonadal mosaicism in the patient and following consequences for pregnancy. Lymphocyte analysis is a standard cytogenetic practice however it cannot address the issue of tissue limited mosaicism, and thus it may not reflect the chromosomal constitution in gonads. The genetic counselling is recommended for the females with detected LLGM.

List of abbreviations:

IVF in vitro fertilisation

ICSI intracytoplasmatic sperm injection

RSA recurrent spontaneous abortions

LLGM low-level gonosomal mosaicism

FISH fluorescent in situ hybridization

Conflict of Interest statement The authors declare that there are no conflicts of interest.

Tab. 1 : Incidence of LLGM and frequency of X aneuploidy in both cohort (significant p<0.05). References

Groups	tested - infertile (N=70)				control (N=83)				P value
mean of age (ranges)	N (patients) 70	mean 30	SD Apr-81	min-max 19-42	N (patients) 83	mean 32.5	SD 4-Sep	min-max 22-46	
incidence of LLGM (chromosomal analysis)	14 (20 %)				11(13.58 %)				$x^2_{(1)}$=0.56288
	frequency of aberrant cells				frequency of aberrant cells				
method	N (patients)	mean(%)	SD	ranges(%)	N (patients)	mean(%)	SD	ranges(%)	
karyo	70	2-Feb	3.743	0-19	83	1-Feb	Feb-37	0-16	0.1346
FISH cultured	66	5-Apr	2.945	1.7-20.0	54	3-Sep	Jan-98	0.8-10	0.002968
FISH uncultured	51	6-Apr	Feb-86	1.7-13.5	46	3-Jun	Jan-64	0-7.6	0.000003
				age<35					
incidence of LLGM (chromosomal analysis)	10(16.12 %)				8(12.12 %)				
	frequency of aberrant cells				frequency of aberrant cells				
karyo	62	Feb-03	Mar-15	0-11.8	66	1-Oct	Jan-82	0-7	0.333
FISH cultured	58	May-28	Mar-18	1.68-20	43	Mar-77	Jan-78	0.81-8.44	0.0003
FISH uncultured	45	6.00	Feb-99	1.74-13.5	37	Mar-57	Jan-73	0-7.6	0.0000
				age>35					
incidence of LLGM (chromosomal analysis)	4 (50 %)				3(17.60 %)				
	frequency of aberrant cells				frequency of aberrant cells				
karyo	8	May-49	Jun-68	0-19	17	Jan-66	Mar-95	0-16	0.097
FISH cultured	8	6-May	Feb-30	2.97-10.05	11	Apr-99	Feb-45	1.76-10	0.129
FISH uncultured	6	8-Nov	Feb-27	4.4-11.0	9	Mar-50	Jan-32	0-4.84	0.0003

Tab. 2: Analysis of clinical data

	without LLGM	with LLGM		
ICSI	control	>1 X aneuploidy cell	> cutoff (FISH)	significancy
n(110)	33	44	33	control x LLGM
Patients with previous spontaneous pregnancies	16 (48.48 %)	20(45.45 %)	14(42.4 %)	ns
Patients with previous spontaneous abortions	6(18.18 %)	9 (20.45 %)	9 (27.27 %)	ns
Patients with reccurrent spontaneous abortions	0	1(2,27 %)	**4 (12,12 %)**	**significant**
Number of ICSI cycles/patient	1	1.5	1.3	ns
Number of oocytes retrieved/ICSI cycle	11	10	9	ns
Number of oocytes suitable for transfer	6	7	6	ns
Clinical pregnancy rate after ICSI	8(24.24 %)	16(36.36 %)	13(39.39 %)	ns
Patients > 35 years	**1(3 %)**	**8(18.18 %)**	**7(21.21 %)**	**significant**
mean age	29	31	31	ns
min - max	24 - 41	24 - 39	24 - 39	ns

Tab.3a: Incidence of LLGM in females - the literature overview

Author	frequency of aberant cells (%)	number of tested patients	method	condition
Capkova	2.20(cyto-cultured)	70	chromosomal	IVF
	5.40(cultured)	66	I- FISH	IVF
	6.40(uncultured)	51	I-FISH	IVF
	1.20 (cyto-cultured)	83	chromosomal	healthy
	3.90 (cultured)	54	I-FISH	healthy
	3.60 (uncultured)	46	I-FISH	healthy
Nowinski et al., 1990	10-Jun	378	chromosomal	RSA
	9-Mar	43	chromosomal	(CHA in child)
	9-Aug	174	chromosomal	healthy
Rusell et al., 2007	1.46(only XCL)	153	chromosomal	RSA
	1.37(only XCL)	330	chromosomal	healthy
Guttenbach et al., 1995	3.1(only XCL)	90	FISH	healthy
Peschka et al., 1999	3.5.(only XCL)	781	chromosomal	ICSI
Patton et al., 2010	2.1 (cultured)	78	FISH	healthy
	0.91 (uncultured)	78	FISH	healthy

RSA - recurrent spontaineous abortions, CHA - chromosomal aberration

Tab.3b: Frequency of X aneuploidy cells in females - the literature overview

Author	frequency of aberant cells (%)	number of tested patients	method	condition
Capkova	2.20(cyto-cultured)	70	chromosomal	IVF
	5.40(cultured)	66	I- FISH	IVF
	6.40(uncultured)	51	I-FISH	IVF
	1.20 (cyto-cultured)	83	chromosomal	healthy
	3.90 (cultured)	54	I-FISH	healthy
	3.60 (uncultured)	46	I-FISH	healthy
Nowinski et al., 1990	10-Jun	378	chromosomal	RSA
	9-Mar	43	chromosomal	(CHA in child)
	9-Aug	174	chromosomal	healthy
Rusell et al., 2007	1.46(only XCL)	153	chromosomal	RSA
	1.37(only XCL)	330	chromosomal	healthy
Guttenbach et al., 1995	3.1(only XCL)	90	FISH	healthy
Peschka et al., 1999	3.5.(only XCL)	781	chromosomal	ICSI
Patton et al., 2010	2.1 (cultured)	78	FISH	healthy
	0.91 (uncultured)	78	FISH	healthy

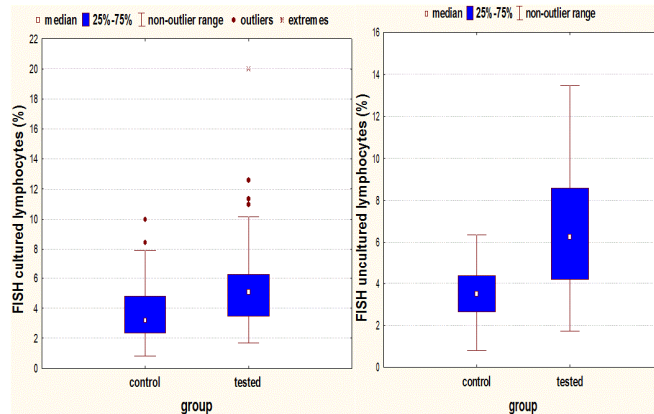RSA - recurrent spontaineous abortions, CHA - chromosomal aberration

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Fig 1: Frequency of X aneuploidy cells by FISH: comparison between control and infertile group by Mann-Whitney test.
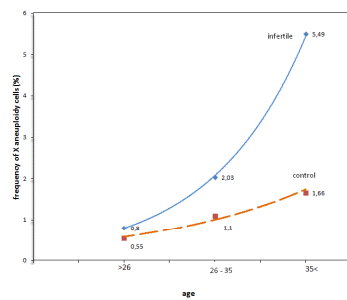

Fig. 2: Correlation of age and frequency of X aneuploidy cells in control and infertile group detected by chromosomal analysis.

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