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STUDY OF ACETYLATED HISTONE H3K9 – AN ACTIVE CHROMATIN MARK – IN CHROMOSOMES FROM ADULT AND FETAL HUMAN LYMPHOCYTES

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❀ **Background.** Incorrect epigenetic modifications of the human genome may result in epigenetic disorders, thus, highlighting the necessity of studying chromosome epigenetic patterns in human development. **Aim of the study:** a comparative analysis of acetylated histone H3K9 (AcH3K9) patterns in human metaphase chromosomes from the lymphocytes of adults and fetuses. **Materials and methods.** The immunocytochemical detection of AcH3K9 in the metaphase chromosomes from PHA-stimulated peripheral lymphocytes of 13 adults and cord blood lymphocytes of 10 fetuses at 20-22 weeks of gestation. **Results.** Both in the chromosomes of the adults and the fetuses, AcH3K9 accumulated in the R- and T-, but not G-bands and avoided the regions of pericentromeric heterochromatin of the chromosomes 1, 9 and 16. When comparing the adult and the fetal chromosomes, different levels of AcH3K9 were revealed in a few bands: 2q31, 5p13, 5p15 and 16p13 had higher level of H3K9 acetylation in adults, in contrast to 9q13 which was hyperacetylated in fetuses. **Conclusion.** The AcH3K9 distribution in metaphase chromosomes is band-specific and is similar between the adults and the fetuses, excluding a few bands with different acetylation levels.

❀ **Keywords:** epigenetics; human development; histone acetylation; chromosomes; chromatin disorders; prenatal diagnosis.

ИЗУЧЕНИЕ МАРКЕРА АКТИВНОГО ХРОМАТИНА — АЦЕТИЛИРОВАННОГО ГИСТОНА H3K9 — ХРОМОСОМ ИЗ ЛИМФОЦИТОВ ЧЕЛОВЕКА В ПРЕ- И ПОСТНАТАЛЬНЫЙ ПЕРИОД ОНТОГЕНЕЗА

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❀ **Введение.** Нарушения эпигенетического маркирования генома приводят к хроматиновым болезням, в связи с чем актуальным представляется изучение эпигенетических маркеров хромосом в онтогенезе человека. **Цель** — провести сравнительный анализ распределения ацетилированного по лизину в 9-м положении гистона H3 (AcH3K9) на метафазных хромосомах из лимфоцитов периферической крови взрослых индивидов и пуповинной крови плодов человека. **Методы.** Иммуноцитохимическая детекция AcH3K9 на препаратах метафазных хромосом из стимулированных фитогемагглютинином лимфоцитов периферической крови 13 взрослых индивидов и пуповинной крови 10 плодов человека 20–22 недель развития. **Результаты.** Участки хроматина, обогащенные AcH3K9, преимущественно локализованы в R- и особенно в T-сегментах метафазных хромосом как у плодов, так и у взрослых индивидов. AcH3K9 отсутствует в блоках прицентромерного гетерохроматина хромосом 1, 9, 16; степень ацетилирования G-сегментов низкая.

Выявлены различия по содержанию AcH3K9 в отдельных сегментах хромосом у взрослых и плодов: в сегментах 2q31, 5p13, 5p15 и 16p13 обнаружен более высокий уровень ацетилирования H3K9 у взрослых индивидов по сравнению с таковым у плодов, а в сегменте 9q13, наоборот, более низкий. **Выводы.** Распределение AcH3K9 вдоль плеч метафазных хромосом из лимфоцитов характеризуется сегментной специфичностью и одинаково у взрослых индивидов и плодов человека за исключением единичных сегментов, в которых уровень ацетилирования H3K9 отличается.

❀ **Ключевые слова:** эпигенетика; онтогенез человека; ацетилирование гистонов; хромосомы; хроматиновые болезни; пренатальная диагностика.

BACKGROUND

The recent identification of a special group of human diseases – epigenetic or chromatin disorders – has raised the interest to study chromatin structure. The term "epigenotype" was introduced for the first time in 1942 to describe the changes of gene expression during development [1]. Currently, epigenetic regulation means the changes of gene expression without structural changes in their nucleotide sequence. Epigenetic modifications establish and control differential gene activity in the cells of different tissues and at different developmental stages. Epigenetic mechanisms provide in the organism phenotypic and functional variety of cells having identical genome [2]. Abnormal epigenetic modifications including the ones caused by the external factors, are the reasons for abnormal genome function, which in turn results in epigenetic disorders, including genomic imprinting diseases: Silver–Russell, Beckwith–Wiedemann, Prader–Willi, Angelman, ICF[1], Rett, Rubinstein–Taybi, and Coffin–Lowry syndromes [3–5].

Epigenetic modifications of chromatin include DNA methylation and posttranslational histone modifications. DNA methylation is a reversible enzymatic reaction, which results in the addition of the methyl group to the fifth position of cytosine residues, mostly in 5'-CpG-3' dinucleotides [6]. Rarely, methylated cytosines are observed in the 5'-CpNpGp-3' and asymmetric 5'-CpA-3' and 5'-CpT-3' sequences [7]. Histone modifications, which include acetylation, methylation, ubiquitination, and phosphorylation, take place mostly in the N-terminal tails. DNA methylation and histone modifications establish specific structural and functional chromatin state, thus, providing epigenetic regulation of its transcriptional activity specific for different cell types and/or stages of ontogenesis [8].

Previous studies have demonstrated that the distribution of methylated regions in metaphase chromosomes coincides with their banding revealed by differential staining in cells from different tissues including peripheral lymphocytes of karyotypically normal adults [9–12], cord blood lymphocytes of human fetuses [13], chorionic cytotrophoblast cells [14, 15], embryonic lung cells [15]. The chromosome methylation pattern is developmental and tissue-specific: the same chromosome bands have different level of DNA methylation in different tissues and at different stages of human ontogenesis [13, 15]. In contrast to the thoroughly studied DNA methylation pattern, the information on the chromosomal distribution of the modified histones is obscure so far.

Here, we compared the chromosomal distribution of acetylated histone H3K9 (AcH3K9), an active chromatin mark, in the lymphocytes of adults and fetuses.

[1] ICF = immunodeficiency, centromeric instability and facial anomalies.

MATERIALS AND METHODS

The metaphase chromosome spreads were prepared from PHA-stimulated peripheral lymphocytes of 13 adults and cord blood lymphocytes of 10 human fetuses at 20–22 weeks. The samples of peripheral and cord blood were obtained from the patients referred to the D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology for postnatal or prenatal karyotyping. The study included only karyotypically normal adults and fetuses.

For culturing, tubes with blood were centrifuged for 1.5 to 2 min at 1000 rpm and leukocyte fraction was taken. Culturing was performed according to the standard protocol [16]. Metaphase chromosomes were fixed using an ethanol solution of 2% glacial acetic acid. The chromosome preparations, without drying, were incubated in 1× phosphate-buffered saline for 5 to 10 min.

Chromosome regions enriched with AcH3K9 were detected immunocytochemically using antibodies against AcH3K9 (Abcam, USA) and the secondary Cy3-conjugated antibodies (Amersham, UK) according to the previously used protocol with modifications [12]. Antibodies were diluted according to the manufacturers' recommendations. For chromosome identification, the AT-specific staining by 4',6-diamidino-2-phenylindole (DAPI) was used.

The chromosome preparations were analyzed using a Leica DMLS microscope equipped with Fluotar ×20/0.40 and ×100/1.30–0.60 objectives, Leica DFC320 color camera, and a set of filter cubes. Photo images were obtained using Leica DFC Twain software. The distribution and intensity of immunofluorescent signals, which reflects the enrichment of chromatin with AcH3K9, were analyzed qualitatively based on visual assessment and, then, quantitatively using ImageJ 1.34s software. ImageJ 1.34s software tools allow to measure the signal intensity in the depicted area of the digital image. Every pixel in the depicted area of the image is automatically assigned the value of 0 to 255 depending on its shade of gray in 8-bit mode. The average fluorescence intensity of the depicted

area is calculated automatically, summing up the values assigned to all pixels and dividing the obtained sum by the number of pixels. Using ImageJ 1.34s software, the absolute values were calculated for the average fluorescence intensity of the chromosome bands. The obtained values were standardized by dividing by the average fluorescence intensity of the benchmark, the chromosome band with constant fluorescence intensity according to visual estimation. The obtained average relative values of fluorescence intensity were compared in Statistica 8.0 using the Mann–Whitney *U*-test.

The chromosomes were not well-spread because the content of acetic acid in the fixative was reduced to 2% in order to prevent the extraction of histone proteins. For this reason, the analysis of the full chromosome set of the cell was impossible and the chromosomal distribution of AcH3K9 was analyzed on metaphase fragments containing 5–15 well-identified chromosomes not overlapping each other. A total of 300 metaphase fragments were analyzed: 180 from adult and 120 from fetal lymphocytes.

RESULTS

The chromosome distribution of AcH3K9 was nonrandom in both adult and fetal lymphocytes. AcH3K9 accumulated in certain chromosome regions with distinct borders thus demonstrating a specific chromosome banding pattern (Fig. 1*a* and *b*). This banding pattern did not show any variation among metaphases.

The borders of AcH3K9-enriched chromatin corresponded to the R/G-band boundaries detected by DAPI. The strongest H3K9 acetylation was detected in DAPI-negative chromosome bands, e. g. R-bands (Fig. 1*c*).

To determine the H3K9 acetylation level, anti-AcH3K9 fluorescence intensity was assessed. Four types of fluorescence signals were distinguished according to the visual assessment of the fluorescence intensity: 0 = absence of fluorescence signal; 1 = very weak fluorescence signal; 2 = weak fluorescence signal; 3 = intense fluorescence signal.

In accordance with this classification, idiograms of acetylated histone H3 localization were designed for all autosomes (Fig. 2).

Strongly acetylated chromatin (3 points) was located in 33 R-bands in both adults and fetuses. Weak anti-AcH3K9 fluorescence (2 points) was present in 62 R-bands in adults and in 59 R-bands in fetuses. A very weak fluorescence signal (1 point), showing chromatin depleted in acetylated histone H3, was detected in all G-bands and in 38 R-bands in adults and in 42 R-bands in fetuses. Fluorescence signal was absent (0 point) in the blocks of pericentromeric heterochromatin of chromosomes 1, 9, and 16 in both adults and fetuses.

The comparative analysis of the anti-AcH3K9 fluorescence intensity between adults and fetuses showed statistically significant difference for a small number of bands: 2q31, 5p13, 5p15, 9q13, and 16p13. Bands 2q31, 5p13, 5p15, and 16p13 had stronger anti-AcH3K9 fluorescence in adults than in fetuses, in contrast to the band 9q13 demonstrating lower fluorescence in adults compared to the fetuses (the Mann–Whitney *U* test, $p \le 0.05$; Fig. 3).

Thus, the chromosomal distribution of AcH3K9 in lymphocytes is band-specific and is similar in adults and fetuses, except for a few bands having different AcH3K9 levels.

DISCUSSION

Histone H3 acetylation marks transcritionally active chromatin [17, 18]. The present study was performed on metaphase chromosomes, for which transcriptional activity is not typical, thus raising the question about the stability of epigenetic patterns throughout the cell cycle. The patterns of some histone post-translational modifications – acetylation of H2AK4, H2BK12, H2BK15, H2BK20, H3K19, H3K23, H4K5, H4K8, H4K12, H4K16 and methylation of H3K27 and H3K36 – undergo changes during the switch of cell cycle phases [19]. However, the level of H3K9 acetylation is stable throughout the cell cycle [20]. Thus, AcH3K9 patterns detected on metaphase chromosomes most probably reflect the potential functional activity of chromatin in the interphase.

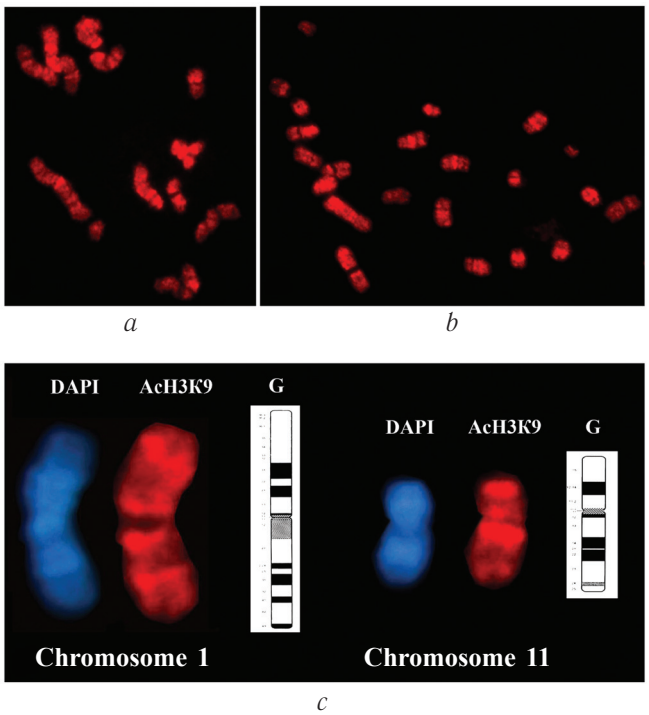

Fig. 1. Fragments of metaphases from the lymphocyte of an adult (*a*) and a human fetus at 21/22 weeks of gestation (*b*) after immunofluorescent detection of acetylated histone H3K9 (AcH3K9). Photo images of chromosomes 1 and 11 of an adult individual after DAPI staining and immunofluorescent detection of AcH3K9, and G-banding ideograms (*c*)

Gene density varies along the chromosome length: it is higher in R-bands than in G-bands [21, 22]. A high gene density in R-bands and a low in G-bands may be associated with the different levels of their transcriptional activity, which, in turn, explains the specificity of AcH3K9 chromosomal distribution observed in our study. According to the obtained data, the degree of R- and T-band acetylation varies. Considering that H3K9 deacetylation results in repression of transcription [23–25], it can be assumed that hypoacetylated R-bands have low transcriptional activity.

Among five bands which are characterized by different levels of H3K9 acetylation in adults and fetuses, 2q31 attracts special attention because it also differs between adults and fetuses by its level of DNA methylation as was detected previously [13]. According to the obtained results, 2q31 is characterized by a high level of H3K9 acetylation and a low level of DNA methylation in the lymphocytes of adults and by a low level of H3K9 acetylation and a high level of DNA methylation in the fetal lymphocytes [13]. Such epigenetic status of 2q31 most probably indicates its lower transcriptional activity in 20–22 week old fetuses compared to that in adults. 2q31 harbors genes of the immunoglobulin superfamily CD51 (*ITGAV*) and CD49D (*ITGA4*) suggesting that the detected levels of DNA methylation and H3K9 acetylation may be associated with the immune system activity in adults rather than in fetuses [26].

In conclusion, the obtained results suggest that the specific chromosomal distribution of AcH3K9 forms a banding pattern in lymphocyte metaphase chromosomes. The variation in acetylation degree of some chromosome bands between fetuses and adults indicates that the re-

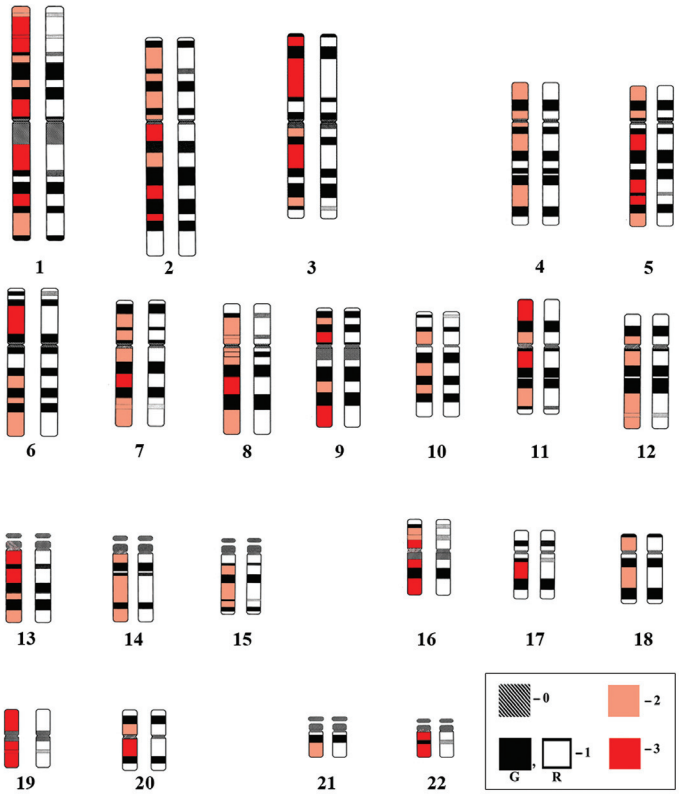

Fig. 2. Band-specific localization of acetylated histone H3K9 (AcH3K9) (*left color idiogram*) in chromosomes from lymphocytes of an adult individual and G-banding (*right black and white idiogram*). Two shades of red show different fluorescence intensity of AcH3K9 in the R-bands – assessed as 2 and 3 points. A low level of fluorescence intensity (1 point) which is typical for all G-bands is shown in black; fluorescence intensity assessed as 1 point in the R-bands is shown in white. Gray color marks the chromosome bands in which fluorescence signal is absent (0 points)

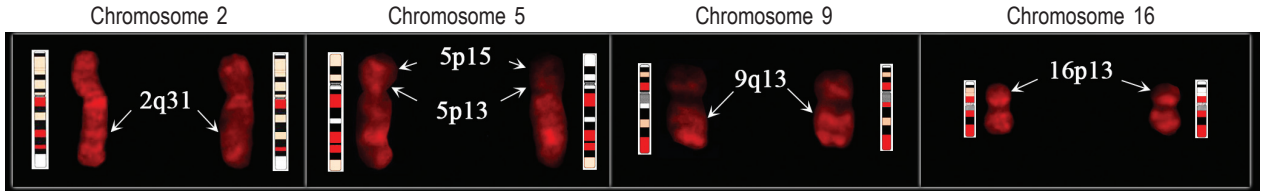

Fig. 3. Distribution of acetylated H3K9 (AcH3K9) along the arms of chromosomes 2, 5, 9 and 16 from lymphocyte of an adult (*left*) and a fetus (*right*). Arrows show the bands with different AcH3K9 level

vealed AcH3K9 pattern reflects not only structural, but also functional chromatin state. It should be noted that genome dysfunction, being the reason for the number of pathological conditions, is the most difficult for diagnostics. Further research of chromosome AcH3K9 patterns together with the transcriptional analysis could become the basis of the test systems for post- and prenatal assessment of chromatin epigenetic status. Importantly, the epigenome plasticity – regulation of epigenetic processes by external effects, including drugs, hormones, and diet – opens horizons for the development of the target-specific therapeutic correction of abnormal epigenetic profiles.

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