Chromatin enrichment of histone marks H4Ac and H3K9me3 in TP53 gene domain in breast cells

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In non-cancerous breast cell lines HB2 and MCF10A the TP53 gene is localized inside a relatively small ~ 50 kb loop domain delimited by two S/MARs. **Aim.** To analyze the chromatin markers H4Ac and H3K9me3 of these two S/MARs and of the TP53 gene P1 promoter in different breast cells lines. **Methods.** We used chromatin immunoprecipitation (ChIP) to characterize the chromatin status of these S/MARs elements in breast non-cancerous cell lines HB2 and MCF10A and cancerous MCF-7, MDA-MB-231, BT-474 and T47D cell lines, by chromatin enrichment of H4Ac and H3K9me3 epigenetic markers, hallmarks of open and closed chromatin, respectively. **Results.** We found that these chromatin epigenetic markers are differentially distributed in S/MARs for all analyzed breast cell lines. **Conclusions.** We found no correlation between S/MARs and chromatin epigenetic status, suggesting that nuclear matrix fixation and chromatin status can be independent. High enrichment of H3K9me3 in the TP53 gene P1 promoter region in MCF-7, could explain lower levels of the TP53 expression, described earlier by our group.

**Keywords:** TP53, loop domain, MAR, breast cancer, chromatin markers, ChIP assay.

**Introduction.** DNA in eukaryotic nucleus is organized in nucleosomes consisting of ~ 147 bp of DNA wrapped around an octamer of four core histone proteins (H2A, H2B, H3 and H4). The N-terminal tail of histones is a subject to several types of post-translational modifications, the two most important of which are acetylation and methylation of some amino acids, usually lysine. These histone modifications can affect the chromatin assembly making it permissible for proteins to modulate cellular processes such as transcription, repair, replication and genome stability [1–4]. Histone acetylation and methylation are catalyzed by histone acetyltransferases (HATs) and histone methyltransferases (HMTs), respectively. Histone H4 acetylation (H4Ac) is a hallmark of open chromatin or euchromatin and may be a sign of gene activation [5–7]. It is generally decreased in breast cancer cell lines and tissues [8, 9]. In contrast, histone H3 lysine 9 trimethylation (H3K9me3) is a hallmark of closed chromatin or constitutive heterochromatin and may denote gene repression [10, 11]. Chromatin is further organized in DNA loop domains delimited by the border elements that include nuclear Scaffold/Matrix Attachment Regions (S/MARs), which could be 20 to 200 kb long [12, 13]. The S/MARs are responsible for DNA attachment to the nuclear matrix (NM) and are linked to DNA replication and transcription. They may include Topoisomerase II binding sites [14, 15] as well as other sequence motifs. Furthermore, NM is enriched in several transcription factors, which have been suggested to be MAR binding proteins (MARBP) [16–18]. Several factors potentially affect the association of DNA with the NM, including the DNA sequence itself and its epigenetic state [19–22]. Scaffold Attachment Regions (SARs) can be enriched in H4Ac [23]. The attachment of human S/MARs to the nuclear matrix de-
pends on a high DNA methylation level and the absence of H3K9ac within S/MAR [24]. Interestingly, some histone modification enzymes such as HATs are associated with NM [25]. Additionally, the loops can be active or inactive due to the MARs chromatin enrichment in H4Ac or H3K9me3, respectively [26].

However, as nuclear matrix remains a controversial concept [13] and the data on the interplay between MARs and histone epigenetic chromatin status are still absent, we decided to test whether the association of S/MARs with the nuclear matrix in breast cancer cell lines corresponds to specific chromatin modifications. We have previously mapped S/MARs in the 167 kb locus of the tumor suppressor gene TP53 in cancerous breast cell lines, MDA-MB-231, BT-474, T47D, MCF-7, and non-cancerous breast cell lines, MCF10A and HB2, using DNA arrays [27]. We found that the loop domain organization in these cell lines was quite variable. MAR2 and MAR3 were detected in non-cancerous cells HB2 and MCF10A, and MAR3 was detected in cancerous cells BT-474 and T47D. Interestingly, these MARs were symmetrically disposed in relation to the TP53 gene P1 promoter, probably constituting the borders of a chromatin loop. Now, in an attempt to check the chromatin accessibility of these two S/MARs and the TP53 gene P1 promoter region we evaluated the chromatin enrichment levels of the histone epigenetic markers H4Ac and H3K9me3 (linked to open and closed chromatin, respectively), by the chromatin immunoprecipitation (ChIP) assay, in the same breast cancerous cells lines. We found no correlation between the chromatin enrichment of histone epigenetic marks and nuclear matrix attachment regions. We believe that our results will help to get a better understanding on the TP53 chromatin domain organization and transcriptional control.

**Materials and methods.** *Cell lines.* All cell lines were obtained from David Cappellen and Nancy Hynes (Friedrich Miescher Institute for BioMedical Research, Novartis Research Foundation, Switzerland). The human mammary carcinoma cell lines MDA-MB-231 and T47D were cultured in DMEM medium supplemented with 10% bovine serum, 0.5% penicillin/streptomycin and 1% glutamine. The human mammary carcinoma cell lines MCF-7 and BT-474 were cultured in RPMI medium supplemented with 10% bovine serum, 0.5% penicillin/streptomycin and 1% glutamine. The control non-cancerous epithelial cell lines MCF10A and HB2 were cultured in DMEM medium supplemented with 10% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 0.5% penicillin/streptomycin and 1% glutamine.

**ChIP assay.** 25·10⁶ cells were cross-linked with 1% formaldehyde for 10 min in a rocking platform at room temperature. Cross-linking reaction was stopped with 0.125 M Glycine diluted in 1× PBS. The cells were washed with ice-cold 1X PBS and resuspended in 1 ml Lysis/Sonication cold buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 7.5) with fresh 0.5 mM PMSF and 1× PIC (Protease Inhibitor Cocktail) and incubated for 30 min on ice. The lysates were homogenized with 10 strokes, centrifuged at 750g for 5 min at 4°C and the supernatant was discarded. The pellet was resuspended in 350 μl of Lysis/Sonication cold buffer with freshly added 1 mM PMSF and 1× PIC, sonicated on ice and centrifuged for 10 min with 2000g at 4°C. The supernatant contains extracted chromatin. The immunoprecipitation was carried out overnight at 4°C with constant agitation in an immunoprecipitation buffer (5 mM Tris-HCl, pH 8.0, 15 mM NaCl, 1 mM EDTA and 0.1% NP 40) with 20 μg of chromatin, 750 μg of Protein G dynabeads #100.03D («Invitrogen®», USA), 4 μg of antibodies H4 pan-acetyl (#39243, «Active Motif®»), trime-thyl H3K9 (#05-1242, «Millipore®»), normal rabbit IgG (#PP64B, «Millipore®»; control) and 1× PIC. All the immunoprecipitations were made at 1:4 dilutions. The magnetic beads were washed 6 times with the immunoprecipitation buffer and the chromatin was eluted with the Lysis/sonication buffer for 15 min at room temperature with constant agitation.

The chromatin was eluted from magnetic beads and reversed cross-linked by 5.25× dilution with 0.2380 M NaCl, denatured at 95°C for 15 min, after 1 μl of RNAse A (10 μg/μl) was added and incubated at 37°C for 15 min and 1 μl of proteinase K (10 μg/μl) was added and incubated at 67°C for 15 min. DNA was cleaned-up by the standard Phenol-Chloroform method and submitted to qPCR. qPCR, was carried out using the standard Syber-Green method with the Mastermix («Roche®», France).

All fold enrichment values of H4Ac and H3K9me3 are relative to the fold enrichment values of the β-actin gene region or the alpha-satellite region, respectively.
All primers, synthesized by SIGMA®, are listed in 5’–3’ and F – forward, R – reverse:

MAR2: F – CTGGCCGGAAATGTTTTCTA, R – GTGCCAGGAGCTGTTCTAGG;
MAR3: F – GTCTGGAGCTATTTGAAAATG, R – TGTCTTCCTGTGCCTGTAGTC;
TP53P1: F – GCGTGTCACCGTCGTGGAAAG, R – GGAGCCTCGCAGGGGTTGATG;
/c98-actin gene region: F – GACGTAGCACAGCCTCTCCT, R – GGGACCTGACTGACTACCTCAT;
Alfa-Satellite (#CS207313, Millipore®) F – C T G C A C T C G A G A T T G G T G T G;
β-actin gene region: F – GACGTAAGCAGCTTCCT, R – GGGACCTGACTGACTACCTCAT;
Alpha-Satellite (#CS207313, Millipore®) F – C T G C A C T C G A G A T T G G T G T G;

All chromatin extraction, immunoprecipitation and qPCR were done in triplicate and the error bars corresponded to the S.E.M. of three different experiments.

Statistic analysis. All statistic data and graphics were performed by the GraphPad version 6.0 using unpaired t-test analyses with Welch’s correction and 95 % confidence interval.

Results and discussion. Association of S/MARs in breast cells is independent of H4Ac and H3K9me3 enrichment levels. Scaffold/Matrix Attachment Regions (S/MARs) have been described as cis-acting factors involved in DNA replication, transcription regulation and chromatin organization. Possibly they take part in the coordination of these cellular processes [28–30]. The tumor suppressor gene TP53 is one of the most studied genes implicated in cancer and, despite a huge knowledge on its post-translational regulation [31, 32], its transcription control and genomic domain are not very well established [33]. In a previous work we described MARs in a genomic region of 167 kb containing the TP53 and neighboring genes, using different cell lines [27]. Here we concentrated our study on two MARs, namely MAR2 and MAR3.

Interestingly, the association of MAR2 with the nuclear matrix was detected only in two analyzed non-cancerous cell lines HB2 and MCF10A where the TP53 gene is organized into a ~ 50 kb loop domain flanked...
by two S/MARs (Fig. 1, A). This organization is perturbed in cancerous cell lines MDA-MB-231, BT-474, T47D and MCF-7 [27]. Chromatin is composed of DNA and proteins, mostly histones, which are involved in genomic DNA organization in the nuclei. Histone alterations in N-terminal tail by acetylation, methylation or ubiquitylation may produce different levels of DNA compaction, leading to an open chromatin or euchromatin, permissive to the protein factors access, or leading to close chromatin or heterochromatin, repressive for the association of transcription factors [34–37]. These histone alterations may be tracked using a ChIP assay, and give an idea of the chromatin ambiance through the epigenetic profile at a genomic site, in normal and cancerous cells [38, 39]. In order to gain insight of the mechanisms governing the attachment of these two S/MARs, MAR2 and MAR3, in normal and breast cancer cell lines and to determine the chromatin state at S/MARs, we performed the ChIP assays to detect the enrichment of both H4Ac and H3K9me3 euchromatin and heterochromatin markers, respectively, in two non-cancerous cell lines: HB2 and MCF10A, and in four cancerous cell lines: MCF-7, T47D, BT-474 and MDA-MB-231 (Fig. 1, B). As presented in Fig. 1, B, we could not identify a specific pattern of the epigenetic histone markers enrichment in non-cancerous and cancerous cells, despite certain differences between histone markers when MAR3 are present in cancerous cells, BT-474 and T47D.

Moreover, the histone enrichment levels were quite different among cell lines, suggesting that neither H4Ac nor H3K9me3, chromatin enrichments, are linked to S/MARs. Indeed, a link between the association of these regions to the NM and active histone marks remains controversial [13, 24, 40]. These results, suggest that nuclear matrix fixation and chromatin epigenetic status can be independent.

Chromatin status of TP53 gene P1 promoter can be linked to TP53 transcription in MCF-7 cells. We have simultaneously performed ChIP assays in the TP53 gene P1 promoter region (TP53P1) to detect chromatin changes associated with the TP53 transcriptional status (Fig. 2). Indeed, in the cell lines, TP53 is less expressed in cancerous cells, relative to HB2, as follows: ~ 2.5X in MCF-7 and T47D, ~ 1.66X in MDA-MB-231 and ~ 1.11X in BT-474 [27]. Interestingly, a higher enrichment of heterochromatin marker H3K9me3 in TP53P1 in MCF-7, as shown in Fig. 2, corroborates the lowest level of the TP53 gene expression observed in this cell line. Also, a higher enrichment of H4Ac in the non-cancerous cell lines, MCF10A and HB2, corroborates higher levels of TP53 described in HB2, in our earlier report [27]. These results together suggest that chromatin status of the P1 TP53 promoter, can regulate the TP53 expression in some breast cell lines.

Conclusions. Our results show no correlation between S/MARs and H4 acetylation/H3K9 trimethylation, suggesting that nuclear matrix fixation and these specific chromatin modifications are possibly independent events during breast cancer progression. Also, chromatin status of the P1 TP53 promoter can regulate TP53 expression in the MCF-7 cell line.

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Збагачення хроматиновими маркерами H4Ac і H3K9me3 домену гена TP53 у клітинних лініях молочної залози

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Резюме

У неонкогенних клітинних лініях HB2 і MSCf10/4 ген TP53 розташований версередній відносно невеликої петлі хроматину (~ 50 тис. пар нуклеоїдів), обмеженою ділом ділянками прикріплення до вдєрного матрикса (ДПМ). Мета. Проаналізувати хроматинові маркери H4Ac і H3K9me3 у зазначених ДПМ і P1 промоторі гена TP53 в різних клітинних лініях молочної залози. Методи. Імунопреципітацію хроматину за допомогою антител проти маркерів активного хроматину H4Ac і гетерохроматину H3K9me3 використано для характеризації стану хроматину елементів ДПМ у неонкогенних клітинних лініях HB2 і MSCf10/4 та зовсімісних клітинних лініях MCF-7, MDA-MB-231, BT-474 і T47D. Результати. Зазначені епігенетичні маркери нерозрізнено розподілені в досліджуваних ДПМ для всіх проаналізованих ліній клітин молочної залози. Висновки. Не знайдено кореляції в епігенетичному статусі ДПМ і хроматину. Це дозволяє припустити, що фіксація у вдєрному матриксе і статус хроматину можуть бути незалежними. Суттєве збагачення H3K9me3 P1 промоторної області гена TP53 клітинної лінії MCF-7 може бути причиною низьких рівнів експресії TP53, описаних раніше нашою групою.

Ключові слова: TP53, хроматин, петлея домен, МАР, рак молочної залози, епігенетичні модифікації гістонів, чин аналізу.

Обговорення хроматиновими маркерами H4Ac і H3K9me3 домена гена TP53 в клітинних лініях молочної залози

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Резюме

В неонкогенних клітинних лініях HB2 і MSCf10/4 ген TP53 розташований в центральній частині петлі хроматину (~ 50 тис. пар нуклеоїдів), обмеженої ділом двома участками прикріплення до вдєрного матрикса (УПМ). Мета. Проаналізувати хроматинові маркери H4Ac і H3K9me3 у зазначених УПМ і P1 промоторі гена TP53 в різних клітинних лініях клітин молочної залози. Методи. Імунопреципітацію хроматину з допомогою антител проти маркерів активного хроматину H4Ac і гетерохроматину H3K9me3 використовували для характеризації стану хроматину елементів УПМ в неонкогенних клітинних лініях HB2 і MSCf10/4 та зовсімісних клітинних лініях MCF-7, MDA-MB-231, BT-474 і T47D. Результати. Зазначені епігенетичні маркери нерозрізнено розподілені в досліджуваних УПМ для всіх проаналізованих ліній клітин молочної залози. Висновки. Не знайдено кореляції в епігенетичному статусі УПМ і хроматину. Це дозволяє припустити, що фіксація у вдєрному матриксе і статус хроматину можуть бути незалежними. Суттєве збагачення H3K9me3 P1 промоторної області гена TP53 клітинної лінії MCF-7 може бути причиною низьких рівнів експресії TP53, описаних раніше нашою групою.

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