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# 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 (MARBPs) [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 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*. $25 \cdot 10^6$ 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 750*g* 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 2000*g* 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®»), trimethyl 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.

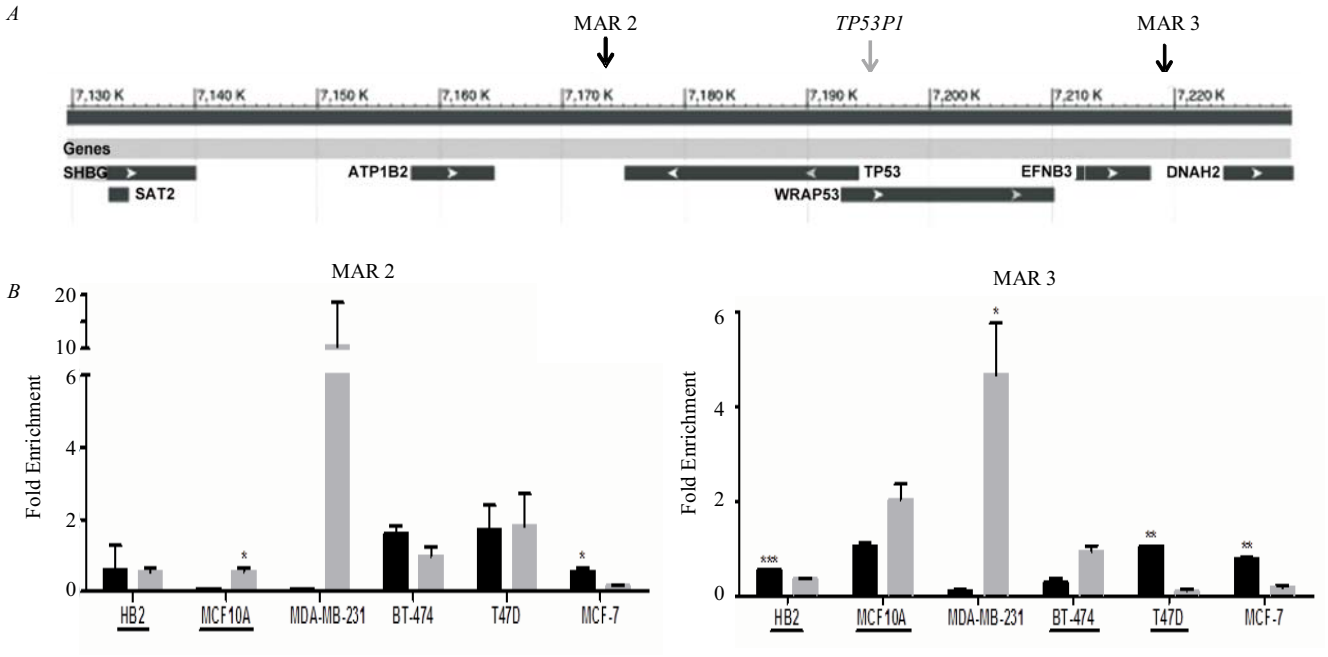


Fig. 1. Chromatin state of MARs in breast cell lines in *TP53* domain: *A* – *TP53* genomic region organization based on NCBI database: NT_010718.16:7.1M-7.2M (94 kb+), covering the 167 kb genomic domain at the 17p13.1 chromosomal region (genes are represented by horizontal bars and arrows indicate the analyzed MARs – black; positions of MAR2 and MAR3 are indicated by black arrows, relatively to position 0, which corresponds to the major transcription start site (P1) of *TP53* (TP53P1, indicated by a gray arrow); in the HB2 and MCF10A cell lines, MARs 2 and 3 delimitate a 50 kb loop encompassing the *TP53*, *WRAP53* and *EFNB3* genes); *B* – ChIP assay of MAR2 and MAR3, indicating the enrichment of euchromatin marker H4Ac (black column)(relative to beta-actin gene region) and heterochromatin marker H3K9me3 (grey column) (relative do alpha-satellite gene region); the cell lines utilized to describe MAR2 or MAR3 are underlined. Error bars correspond to +/–S.E.M. from 3 different experiments; *p < < 0.05; **p < < 0.005; ***p < 0.0001

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;

β-actin gene region: F – GACGTAGCACAGCT TCTCCT, R – GGGACCTGACTGACTACCTCAT;

Alpha-Satellite (#CS207313, Millipore®) F – CTG CACTACCTGAAGAGGAC, R – GATGGTTCAAC ACTCTTACA.

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

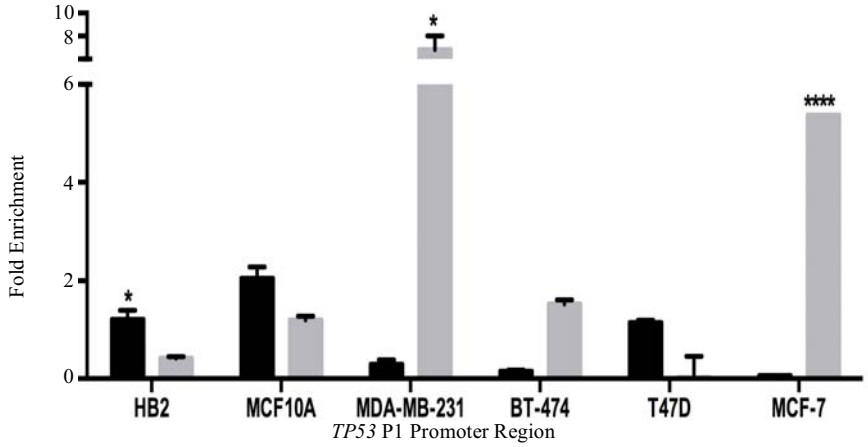


Fig. 2. Chromatin state of *TP53* P1 Promoter region. ChIP assay of the *TP53* P1 promoter region, indicating the enrichment of euchromatin marker H4Ac (black column) (relative to beta-actin gene region) and heterochromatin marker H3K9me3 (grey column) (relative do alpha-satellite gene region). Error bars cor respond to +/– S.E.M. from 3 different experiments; *$p < 0.05$; ****$p < 0.00001$

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 H3K9 me3 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 mar kers 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 MCF10A ген TP53 розташований всередині відносно невеликої петлі хроматину (~ 50 тис. пар нуклеотидів), обмеженої двома ділянками прикріплення до ядерного матриксу (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 антитіл проти маркерів активного хроматину H4Ac і гетерохроматину H3K9me3 використано для характеристики стану хроматину елементів ДПМ у неонкогенних клітинних лініях HB2 і MCF10A та злоякісних клітинних лініях MCF-7, MDA-MB-231, БТ-474 і T47D. **Результати**. Зазначені епігенетичні маркери нерівномірно 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 ядерному матриксі і статус хроматину можуть бути незалежними. Суттєве збагачення H3K9me3 P1 промоторної області гена TP53 клітинної лінії MCF-7 може бути причиною нижчих рівнів експресії TP53, описаних раніше нашою групою.*

*Ключові слова: TP53, хроматин, петлевий домен, MAP, рак молочної залози, епігенетичні модифікації гістонів, чип аналізу.*

Обогащение хроматиновыми маркерами H4Ac и H3K9me3 домена гена *TP53* в клеточных линиях молочной железы

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Резюме

*В неонкогенных клеточных линиях HB2 и MCF10A ген TP53 расположен внутри относительно небольшой петли хроматина (~ 50 тыс. пар нуклеотидов), ограниченной двумя участками прикрепления к ядерному матриксу (УПМ). **Цель**. Проанализировать хроматиновые маркеры H4Ac и H3K9me3 в указанных УПМ и P1 промоторе гена TP53 в различных клеточных линиях клеток молочной железы. **Методы**. Иммунопреципитацию хроматина с помощью антител против маркеров активного хроматина H4Ac и гетерохроматина H3K9me3 использовали для характеристики состояния хроматина элементов УПМ в неонкогенных клеточных линиях HB2 и MCF10A и злокачественных клеточных линиях MCF-7, MDA-MB-231, БТ-474 и T47D. **Результаты**. Указанные эпигенетические маркеры неравномерно распределены в исследованных УПМ для всех анализируемых линий клеток молочной железы. **Выводы**. Не выявлена корреляция в эпигенетическом статусе УПМ и хроматина. Это позволяет предположить, что фиксация в ядерном матриксе и статус хроматина могут быть независимыми. Существенное обогащение H3K9me3 P1 промоторной области гена TP53 клеточной линии MCF-7 может быть причиной более низких уровней экспрессии TP53, описанных ранее нашей группой.*

*Ключевые слова: TP53, хроматин, петлевой домен, MAP, рак молочной железы, эпигенетические модификации гистонов, чип анализа.*

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