TIP60/P400/H4K12ac Plays a Role as a Heterochromatin Back-up Skeleton in Breast Cancer

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Abstract. *Background/Aim:* In breast cancer, initiation of carcinogenesis leads to epigenetic dysregulation, which can lead for example to the loss of the heterochromatin skeleton SUV39H1/H3K9me3/HP1 or the supposed secondary skeleton TIP60/P400/H4K12ac/BRD (2/4), which allows the maintenance of chromatin integrity and plasticity. This study investigated the relationship between TIP60, P400 and H4K12ac and their implications in breast tumors. *Materials and Methods:* Seventy-seven patients diagnosed with breast cancer were included in this study. Chromatin immunoprecipitation (ChIP) assay was used to identify chromatin modifications. Western blot and reverse transcription and quantitative real-time PCR were used to determine protein and gene expression, respectively. *Results:* We verified the variation in H4K12ac enrichment and the co-localization of H4K12ac and TIP60 on the euchromatin and heterochromatin genes, respectively, by ChIP-qPCR and ChIP-reChIP, which showed an enrichment of H4K12ac on specific genes in tumors compared to the adjacent healthy tissue and a co-localization of H4K12ac with TIP60 in different breast tumor types. Furthermore, RNA and protein expression of TIP60 and P400 was investigated and overexpression of TIP60 and P400 mRNA was associated with tumor aggressiveness. *Conclusion:* There is a potential interaction between H4K12ac and TIP60 in heterochromatin or euchromatin in breast tumors.

Breast cancer is the most frequent cancer (24.2% new cases in 2018) and the leading cause of cancer-related death (15% in 2018) (1). The majority of breast cancers are sporadic (non-hereditary), representing 80 to 90% of breast cancers (2). Dereglulation of chromatin integrity and plasticity is among the causes of sporadic cancer (3). They occur at the epigenetic level in the different compartments of chromatin, which are heterochromatin (compacted chromatin) that inhibits gene expression, and euchromatin (relaxed chromatin) that promotes gene transcription. These chromatin states are maintained by so-called epigenetic proteins, which, by placing groups or marks on the histones, allow entry or exit from one state to another (4-6).

Among these proteins, SUV39H1, which is a histone methyltransferase (HMT) that adds the H3K9me3 marker on the site where the HP1 protein will bind, thus forming the SUV39H1/H3K9me3/HP1 skeleton which keeps the chromatin closed and inhibits transcription in the heterochromatin region (7, 8). Heterochromatin houses inactive genes such as numerous oncogenes; the loss of this structure would lead to the transcription of oncogenes. Several studies have shown a loss of the SUV39H1 protein in cancer, but also a loss of the SUV39H1/H3K9me3/HP1 skeleton during the onset of carcinogenesis (7, 9).

The acetyl-transferases (HAT) such as TIP60 are able to add acetyl groups on the N-terminal tails of histones resulting in the opening of the chromatin leading to euchromatin (10). TIP60 is also capable of acetylating non-histone proteins such as p53 and ATM allowing their activation (11-13). Many studies have shown that histone acetylation by TIP60 is observed in euchromatin and heterochromatin, allowing the maintenance of these structures (14). Indeed, TIP60 can be recruited at the heterochromatin due to its chromo-domain capable of binding to H3K9me3 or H3K9me groups (15), but can also be recruited via its complex with the H2Az variant into the heterochromatin by the ATPase P400 (16, 17). This recruitment would compensate for the loss of the heterochromatin maintenance

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skeleton (SUV39H1/H3K9me3/HP1) by the overexpression of histone demethylase. Grézy *et al.* found that treatment of SUV39H1/2 and NIH3T3 cells with azacytidine resulted in an enrichment of H4K12ac, TIP60, P400 and BRD2 on the heterochromatin (16). This enrichment revealed the existence of a back-up skeleton for the heterochromatin that comprised TIP60/ H4K12ac/BRD2. Indeed, bromo-domain proteins such as BRD2 and BRD4 are able to bind to the H4K12ac mark preventing the detachment of chromatin (18). In breast cancer, a decrease in the protein levels and a residual activity of TIP60 has been observed (19).

In this study, we hypothesized that the H4K12ac modification in heterochromatin and euchromatin is realized by TIP60 and its complex especially with P400, thus allowing maintenance of chromatin stability and integrity. This recruitment of TIP60 could explain its presence in heterochromatin and thus, the dysregulation of the acetylation of p53 and tumor suppressors.

The study of this residual activity of TIP60 in the maintenance of chromatin structures will allow a better understanding of its role in the aggressiveness of cancers and especially of breast cancer.

Materials and Methods

Clinical samples. This study included 77 patients admitted to the Centre Jean Perrin from September 2008 to February 2019, and diagnosed with breast cancer (Table I). Patients were informed about the study and gave informed consent prior to inclusion. All 77 tumors and their adjacent normal breast tissues were obtained from the Centre Jean Perrin tumor bank, Biological Resource Center (CRB), accredited under No.BB-0033-00075, where they were stored in liquid nitrogen at -196°C . The 77 patients selected did not receive chemotherapy and/or radiotherapy, were not predisposed to breast cancer and had no family members with breast cancer.

Chromatin immunoprecipitation (ChIP) assays and Quantitative real-time PCR method and data analysis. ChIP assays were performed on chromatin extracted from breast tumors using the Auto iDeal ChIP-seq kit for Histones (C01010171, Diagenode, Seraing, Belgium) according to manufacturer's instructions and the experimental setup developed in our laboratory (20, 21). The antibodies used were 3 μg of anti-H4k12ac Abs (C15200218, Diagenode) and 1 μg non-immune rabbit IgG (Kch-504-250, Diagenode) serving as a negative control.

For Re-ChIP assays, the immunoprecipitated DNA from the first ChIP assay was used. The second ChIP assay (re-ChIP) was then carried out using 3 μg of anti-TIP60 Abs (SC-166323, Santa Cruz Biotechnology, Dallas, TX, USA) according to the experimental setup developed in our laboratory (20, 21). The quality control and efficacy of all ChIP assays performed in this study were verified using positive and negative controls provided in the manufacturer's kit and according to their instructions (Diagenode). Control of ChIP analysis was performed prior to direct H4K12ac ChIP assays and prior to H4K12ac and TIP60 ChIP and re-ChIP assays.

Real-time qPCR was performed in triplicate at 25 μl final reaction volume that included 5 μl of IP or input, 1 $\times$ de SYBR®

Table I. Characteristics of the breast cancer patients included in this study.

	Age		Grade			Ki67	
	<50	>50	I	II	III	≤ 20	>20
Luminal A	2	26	7	13	8	18	10
Luminal B	1	27	19	5	4	17	9
TNBC	1	20	0	6	15	4	17

Green PCR Master Mix (Applied Biosystems, CA, USA), 200 nM for each GAPDH (C17011047, Diagenode) and Myoglobin Exon 2 primers (C17011006, Diagenode), on a 7900 HT Fast Real Time PCR System (Applied Biosystems).

Protein extraction and immunoblot analysis. Breast cancer tumors and their adjacent healthy tissues were crushed with the crusher (french press) and protein extracts were obtained using NucleoSpin® RNA/Protein, Mini kit for RNA and protein purification (Macherey-Nagel, Hoerd, France). About 25–40 μg of extracted proteins were resolved by electrophoresis on 8–15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA, USA). Electro-transfer, blockage of membranes, immunoblotting and immunolabeling were performed using the manufacturer's protocol and the experimental methods developed in our laboratory (20, 22). The primary antibodies (Abs) used were: anti-TIP60 Abs (1/500, GTX112197) and anti-P400 Ab (1/500, GTX116689) were purchased from GenTex (CA, USA), anti-H4k12ac Abs (1/750, C15200218, Diagenode) and anti- GAPDH Abs (1/5000, sc-47724, Santa Cruz Biotechnology). The secondary Abs: anti-mouse IgG (1/2000, S3721) and anti-rabbit IgG (1/2000, S3738) were purchased from Promega (Madison, WI, USA).

RNA extraction, reverse transcription (RT) and quantitative real-time PCR analysis. Breast tumors and their adjacent healthy tissues were crushed at the french press. RNA extracts were obtained using NucleoSpin® RNA/Protein, Mini kit for RNA, protein purification (Macherey-Nagel, France) and reverse transcription were performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and Synthesized cDNAs were amplified using TaqMan Gene expression PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol and the experimental methods developed in our laboratory (20, 22). The TaqMan Gene Expression assay-on-demand used were TIP60 [Hs00197310_m1] or P400 [Hs01566078_m1] and endogenous control 18S Rrna [Hs99999901_s1].

Statistical analyses. Relative expression levels of TIP60, p400 and H4K12ac protein assayed by immunoblotting were quantified using ImageJ software. Data analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was calculated by One-Way ANOVA test with a multiple comparison test to assess statistical significance between groups. A p -value <0.05 (*) was considered significant, $p<0.01$ (**) very significant and a $p<0.001$ (***) highly significant, as stated in the figure legends.

Figure 1. Fold enrichment in H4K12ac over IgG (illustrating the presence of H4K12ac on promoters in euchromatin genes (*GAPDH* and *ADH5*) and in heterochromatin (*Myoglobin*, *SATα* and *SAT 2* genes) in adjacent healthy breast tissues and Luminal A, Luminal B and TNBC breast tumors. Results are expressed as fold increase $\pm$ SD of ($N=19$, $N=19$ and $N=12$, for Luminal A, Luminal B and TNBC, respectively). p -values were calculated by two-tailed students t -test (* indicating $p<0.05$, ** $p<0.01$ and *** $p<0.001$). Black bar: breast tumors and white bar: healthy breast tissues.

Results

H4K12ac enriched on genes in euchromatin and heterochromatin in breast tumors. To observe H4K12ac enrichment on the promoters in euchromatin (*GAPDH* and *ADH5*) and heterochromatin (*Myoglobin*, *SATα* and *SAT2*) genes in breast cancer, a ChIP-qPCR was performed on 19 Luminal A, 19 Luminal B and 12 triple negative breast cancer (TNBC) tumors and their adjacent healthy tissues (Figure 1). An enrichment of H4K12ac was found in different euchromatin and heterochromatin genes in almost equal proportions in healthy breast tissues (Figure 1), whereas this enrichment was significantly increased in Luminal A and B tumors compared to their adjacent healthy tissues (Figure 1). In TNBC tumors, this enrichment increased slightly compared to healthy tissues, and a decrease in enrichment can be seen

in TNBC compared to Luminal tumors (Figure 1). These results suggest that there is an equal distribution of H4K12ac in the different chromatin compartments in healthy breast tissues, but this marker is highly increased in Luminal tumors and slightly in TNBC tumors.

Co-localization of H4K12ac and TIP60 in breast tumors. To determine whether TIP60 is responsible for the H4K12ac modification, we performed ChIP-reChIP and examined whether they co-localized in euchromatin (*GAPDH* and *ADH5* genes) and in heterochromatin (*Myoglobin*, *SATα* and *SAT 2* genes) (Figure 2). A significant high co-localization was obtained in healthy tissues compared to tumors and this co-localization decreased significantly in luminal A and TNBC tumors. In luminal B tumors, the co-localization of H4K12ac and TIP60 was more important than

Figure 2. Co-localization of H4K12ac and TIP60 in promoter regions of target genes on euchromatin (GAPDH and ADH5) and heterochromatin (Myoglobin, SATα and SAT 2) in adjacent healthy breast tissues and breast tumors Luminal A, Luminal B and TNBC. Results are expressed as percentage $\pm$ SD of six tumor and healthy tissues. *p*-values calculated by two-tailed students *t*-test (* indicating $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Black bar: breast tumors and white bar: healthy breast tissues.

in other types of tumors. This could suggest that TIP60 is recruited during the carcinogenesis induction, because in the less aggressive tumors there is an increase in H4K12ac mark and co-localization of TIP60 and H4K12ac from Luminal A to Luminal B tumors. This recruitment is lost in the most aggressive TNBC tumors, which could be due to the high decrease in TIP60 expression in these aggressive tumors.

Variations in the expression of TIP60 and P400 in breast tumors. After observing the variations in the enrichment of

the H4K12ac mark and the co-localization of TIP60 with this mark, TIP60 and P400 mRNA and protein accumulation in breast tumors and adjacent healthy tissues was examined. RNA expression was studied by RT-qPCR, which demonstrated a significantly lower expression of the mRNA of TIP60 and P400 in Luminal tumors, while a significant overexpression of TIP60 and P400 mRNA was observed in TNBCs tumors (Figure 3). The expression of TIP60 and P400 proteins as well as the H4K12ac modification in breast tumors and their adjacent healthy tissues were studied by

Figure 3. Relative RNA expression of TIP60 and P400 of breast tumors (Luminal A, Luminal B and TNBC) compared to their healthy adjacent tissues. Results are expressed as relative expression $\text{Log}_{10} \pm \text{SD}$ of triplicates, the significance is represented by (*) if the expression is twice more or less important or (**) if the expression is ten times more or less important). Black bar: TNBC breast tumors, grey bar: Luminal B breast tumors and white bar: Luminal A breast tumors.

western blot (Figure 4). A decrease in protein and mark levels between tumors and healthy tissues were observed. These observations demonstrated a difference in the transcription and translation of TIP60 and P400. In fact, transcription of TIP60 and P400 was decreased in the least aggressive tumors (Luminal) and increased in the most aggressive tumors (TNBC) (Figure 3). However, their translation into proteins progressively decreased with the aggressiveness of the tumor (Figure 4). This may suggest that there is a deregulation of the translation of these proteins confirming the decrease in the co-localization between H4K12ac and TIP60 observed by re-ChIP.

Discussion

In breast cancer, downregulation of TIP60 is observed early in carcinogenesis (19, 23), which seems to affect the activities of TIP60 in cancer. TIP60 is a pleiotropic protein that plays a role in several cellular mechanisms involved in tumor development and growth due to its ability to acetylate non-histone proteins such as p53 and ATM (12, 13, 24).

Recruitment of TIP60 in heterochromatin to compensate for the loss of the SUV39H1/H3K9me3/HP1 skeleton, in early carcinogenesis, may decrease the activation of tumor suppressors such as p53 (24-26). To confirm this recruitment, we first examined the enrichment of H4K12ac in heterochromatin and euchromatin. We found that the levels of H4K12ac in non-tumor tissues did not vary across chromatin species (Figure 1). However, in tumors, particularly luminal tumors, there was a strong increase in H4K12ac levels, which were similar to those in healthy tissues of TNBC tumors (Figure 1). Knowing that H4K12 is a target for TIP60 (27), we investigated the co-localization of H4K12ac and TIP60 in the different chromatin species in breast tumors.

High co-localization of H4K12ac and TIP60 was observed in healthy tissues compared to tumors (Figure 2). However, a decrease in co-localization of H4K12ac and TIP60 was observed in Luminal A and TNBCs, whereas in intermediate Luminal B tumors there was an increase in this co-localization (Figure 2). These results could suggest that TIP60 was already present in the different chromatin species to deposit the H4K12ac mark because there was a higher co-localization of H4K12ac and TIP60 in the corresponding healthy tissues compared to the tumors. However, the increased levels of H4K12ac and the co-localization with TIP60 observed in Luminal B tumors compared to Luminal A and TNBCs could be the result of a concentration or recruitment of TIP60 to compensate for the progressive loss of the SUV39H1/H3K9me3/HP1 skeleton. This loss of H4K12ac enrichment and co-localization with TIP60 in TNBCs is due to the progressive loss of TIP60 protein expression in these tumors observed by western blotting (Figure 4). The loss of TIP60 protein during breast cancer progression has been demonstrated (23). Under-expression of TIP60 and P400 mRNA was observed in Luminal tumors and over-expression in TNBCs (Figure 3). This could suggest that the increased expression of TIP60 RNA was disrupted at the translational level, resulting in reduced levels of the protein. This study showed that during carcinogenesis, TIP60 is highly solicited in heterochromatin but also in euchromatin probably to maintain chromatin stability and integrity. During the progression and depending on the aggressiveness of carcinogenesis, translational perturbation of TIP60 leads to its loss but also to the loss of the TIP60/P400/H4K12ac skeleton and chromatin instability.

To better understand this mechanism (Figure 5), TIP60 or P400 depletion could be induced *in vitro* in different breast cancer and non-cancer cell lines. This will allow us to examine how H4K12ac levels vary in the different species of chromatin after this depletion. The depletion of TIP60 or P400 will also allow us to investigate how TIP60 acts on P400 and *vice versa*.

Figure 4. TIP60, P400, H4K12ac and GAPDH protein expression in the different breast tumors (Luminal A, Luminal B and TNBC) and their corresponding healthy adjacent tissues. (a) Shows the immunoblot of extracts from Luminal A breast tumors and their corresponding healthy tissues. (b) Shows the immunoblot of extracts from Luminal B breast tumors and their corresponding healthy tissues. (c) Shows the immunoblot of extracts from TNBC tumors and their corresponding healthy tissues. Quantification was performed by Image J.

Figure 5. Illustration of the variation of H4K12ac enrichment as a function of tumor aggressiveness, protein expressions of TIP60 and P400 but also the expression of TIP60 and P400 RNA in heterochromatin and euchromatin.

Conclusion

In this study, the presence of TIP60 and H4K12ac modification was confirmed and a decrease of these two proteins was observed in breast tumors compared to healthy tissue. TIP60 and H4K12ac are present in euchromatin and heterochromatin. Finally, interaction between TIP60, P400 and H4K12ac was observed. The understanding of TIP60/P400/H4K12ac skeletal formation, the variations in its different components and the impact they have on each other will allow us to better understand carcinogenesis. This will undoubtedly allow us to use TIP60 or H4K12ac as a biomarker for the aggressiveness of the tumor, but also to use TIP60 as a therapeutic target against breast cancer.

Conflicts of Interest

There are no conflicts of interest to declare in relation to this study.

Authors' Contributions

Writing-review & editing, Mouhamed Idrissou; Methodology, Tiphane Boisnier, Anna Sanchez and Fatma Zohra Houfah Khoufah; Resources, Frédérique Penault-Llorca, Yves-Jean Bignon.; Supervision, project administration, Writing-review & editing, Dominique Bernard-Gallon.

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