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Research article

Analysis of SATB1 And SATB2 Expression in The Mouse Model of Chemically Induced Skin Carcinogenesis

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ABSTRACT
Special AT-rich sequence binding proteins (SATB) 1 and the closely related SATB2 have been proposed to act as genome organizers that regulate chromatin structure and gene expression by recruiting chromatin remodeling/modifying enzymes and transcription factors to genomic DNA. Despite the fact that the changes in the expression levels of SATB1 and SATB2 were shown to be associated with tumor growth and metastasis development in various cancer cells such as lymphoma, colorectal and breast cancer cells, the potential role of SATB1 and SATB2 gene activity in tumors of the skin is still unknown. In the present study, SATB1 and SATB2 expression levels were investigated in mouse skin at early and middle stages of chemically induced carcinogenesis by quantitative RT-PCR analysis. Here, it was found that both SATB1 and SATB2 were down-regulated during the middle stage (papillomas) of skin carcinogenesis. Furthermore, the comparison of the relative expression levels of SATB1 to SATB2 has shown that SATB2 has a greater down-regulation in the middle stage of skin carcinogenesis. These data provide a fundamental knowledge and insight about SATB1 and SATB2 association with the skin carcinogenesis by determining and comparing their relative gene expression levels.

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Introduction

Mammalian skin is composed of at least three differentiating epithelial compartments: the epidermis, the hair follicle and the associated glands (e.g. sebaceous glands) [1]. The growth of epidermis and sebaceous glands is continuous, while hair growth is cyclic [1]. In all continually renewing tissues, a stem cell population is present to provide a source of differentiating cells [2]. In mouse skin, an epithelial stem cell population is thought to localize to the bulge region of the hair follicle at the arrector pili muscle attachment site, the segment that does not undergo regression during the hair cycle [3]. It has been shown that the hair follicles play an important role in mouse models of chemically induced skin carcinogenesis [4] and topical application of a carcinogen at specific phases of the hair cycle results in striking differences in tumor formation [4].

Tissue development and homeostasis are controlled by establishing specific gene expression programs in defined populations of stem cell progeny. Epidermal stem cells have been classically characterized as slow-cycling, long-lived cells that reside in discrete niches in the skin [1]. Stem cells (SCs) in the epidermis have a crucial role in maintaining tissue homeostasis by providing new cells to replace those that are constantly lost during tissue turnover or following injury [5]. Different resident skin stem cell pools contribute to the maintenance and repair of the various epidermal tissues of the skin, including inter-follicular epidermis, hair follicles and sebaceous glands. Interestingly, the basic mechanisms and signaling pathways that orchestrate epithelial morphogenesis in the skin are reused during adult life to regulate skin homeostasis [5]. Adult SCs may be the initial target cells, as they self-renew for extended periods of time, providing increased opportunity to accumulate the mutations required for cancer formation [6].

Interestingly, cancer stem cells derived from epidermal tumors exist independent of the classic skin stem cell niche, yet also have stem cell properties [7]. Epidermal cancer stem cells are thought to be rapid-cycling cells and exist in the absence of normal niche signals, yet it has been postulated they still retain the capacity for multi-lineage differentiation [7]. New evidence suggests that in the mouse, cancer stem cells are reliant on expression of the cell surface antigen, CD34, and intact Wnt/β-catenin signaling [7]. Evidence suggests that

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the p63 and c-Myc networks are likely to be involved [7]. Human cancer stem cells are thought to express high levels of the cell surface marker, CD44, but have reduced levels of Lrig1 and MAP4, two proteins that putatively regulate quiescence in normal human epidermal stem cells [7].

Special AT-rich sequence-binding protein 1 (SATB1) is a nuclear protein that functions as a genome organizer essential for proper T-cell development and expression of the Th-2 cytokine locus [8]. SATB1 is the most well-characterized matrix attachment region (MAR) binding protein (MBP) that participates in the maintenance and compaction of chromatin architecture by organizing it into distinct loops via periodic anchoring of MARs to the nuclear matrix [9]. SATB1 constitutes a functional nuclear architecture that has a 'cage-like' protein distribution surrounding heterochromatin. This architecture is referred to as 'the SATB1 regulatory network', as SATB1 regulates gene expression by recruiting chromatin remodeling or modifying enzymes and transcription factors to genomic DNA, which it tethers via base-unpairing regions (BURs) [10]. SATB1 also acts as a 'docking site' for several chromatin modifiers including ACF, ISWI, and HDAC1 [10] and these chromatin modifiers were suggested to suppress gene expression through histone deacetylation and nucleosome remodeling at SATB1-bound MARs [10].

Special AT-rich sequence-binding protein 2 (SATB2) has high structural similarity to SATB1 and can also bind to AT-rich DNA sequences [11] and interact with chromatin remodeling complexes [12]. Mouse studies have shown that SATB2 has a key role in B-cell differentiation [13], in osteoblast differentiation [14], in the establishment of neural corticocortical connections across the corpus callosum [15], in the developing jaw and incisors [16] and in developmental regulation of neuronal differentiation [11]. Recently, a loss-of-function study in the mouse has demonstrated that SATB2 is essential for proper facial patterning of the embryo and for normal bone development [11, 14] and also the Inactivation of the SATB2 gene by homologous recombination leads to perinatal lethality because of the multiple cranio-facial abnormalities [17].

The nuclear matrix (NM) is an elusive structure according to many studies and its existence and structure has long been questioned [18]. It has been described as a dynamic sponge with open compartments for free diffusion in the nucleoplasm [19] and also it has been argued that the NM structure is an artefact generated by the sample preparation [20]. Perhaps the NM can best be described as a structural component inside the nucleus, to which chromatin binds via matrix (or scaffold) attachment regions of the DNA forming looped chromatin structures [21]. These specialized genomic sequences possessing high affinity for the nuclear matrix and also for various chromatin architectural proteins are termed matrix attachment regions (MARs) and are utilized in a selective and dynamic manner to tether chromatin loops in vivo [22]. MARs have been implicated in the regulation of transcription by altering the organization of eukaryotic chromosomes and augmenting the potential of enhancers to act over large distances [22]. They are frequently located at the boundaries of transcription units where they are likely to delimit the ends of the active chromatin domains in terms of transcription as well as replication [21]. The MAR sequences commonly contain regions where base pairs tend to break under an unwinding stress (base-unpairing region (BUR)), which is important in binding to the nuclear matrix [23]. Chromatin architecture plays an important role in the regulation of nuclear function [24]. Since the chromatin structure and dynamics are crucial in gene regulation, miss-regulation of gene expression caused by the defects in chromatin remodeling leads to numerous diseases including cancer [24].

SATB1 is not expressed in all cells and it seems particularly important in cells that must change their function – as many progenitor cells do, including the thymocytes that turn into T cells, and as cancerous cells must do to turn into metastatic cells [25]. In most recent studies, it was shown that SATB1 is over-expressed in breast cancer cells and it coordinates expression of a large number of genes to induce metastasis [26]. SATB1 was also found to be highly expressed in multidrug-resistant breast carcinoma cell lines and tissues with P-glycoprotein (Pgp) over-expression [27]. Removal of SATB1 from aggressive breast cancer cells not only reverse metastatic phenotypes but also inhibit tumor growth, indicating its key role in breast cancer progression [26]. SATB1 is overexpressed in many cancer cells, such as colorectal cancer cells where it is associated with tumor progression, invasion, and metastasis [27]. In lymphomas, SATB1 plays a role in T-cell development and differentiation, particularly in aggressive subtypes like T-cell lymphomas. Its expression is linked to increased proliferation and survival of malignant lymphocytes [28]. SATB1 also correlates with high expression of tumor necrosis factor (TNF)-a, which mediates the inflammatory processes in tumor invasion, angiogenesis, and metastasis [27]. On the other hand, SATB1 influences the expression of anti-inflammatory interleukin (IL)-4 and IL-10, which in turn are known to lead to escape from cancer immune surveillance [28]. SATB1 also suppresses the apoptosis rate, which is known to allow cells to escape from chemotherapy [27].

It has been also found that low expression of SATB2 is correlated with tumor progression and poor prognosis in patients with colorectal cancer (CRC), suggesting that SATB2 is a novel potential prognostic marker for CRC [29]. In osteosarcoma, SATB2 promotes osteogenic differentiation, and its expression is linked to tumor progression and aggressiveness by enhancing cell proliferation and invasion [30]. Similarly, in prostate cancer, SATB2 is implicated in tumor progression and resistance to therapy possibly through regulating genes involved in cell survival and metastasis [31]. Additionally, in Gastric and Esophageal Cancers, SATB2 downregulation is associated with poor prognosis and increased tumor invasiveness [32].
While high SATB1 expression is linked to tumor growth and metastasis in breast cancer, colorectal cancer, and lymphoma, its role in other tissues, including skin, remains unknown. Similarly, the role of SATB2 in most tumors is also still remains unclear [26]. Therefore, the main aim of this study is the analysis of SATB1 mRNA expression and SATB2 mRNA expression at different stages of tumor development in the mouse model of chemically induced skin carcinogenesis using qRT-PCR technique.
Mouse skin has provided an excellent paradigm for studies of multistage chemical carcinogenesis in epithelial cells [33]. The most common chemical carcinogenesis regimen is two stage induction, which involves the administration of a single dose of the polycyclic aromatic hydrocarbon 7, 12 - dimethylbenz[a]anthracene (DMBA), followed by weekly applications of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) [33]. This treatment results in the development of numerous benign papillomas, some of which progress to malignant squamous cell carcinomas in 20 to 40 weeks after the first exposure to carcinogens [33]. This common chemical carcinogenesis regime (two-stage induction) closely mimics the multistep process of human skin carcinogenesis, including initiation, promotion, and progression, making it a well-established model for studying tumor development and molecular changes in the skin. By inducing carcinogenesis in mouse skin, SATB1 and SATB2 expressions during the different stages of the tumor formation can be investigated to reveal their possible roles in skin cancer.

Material and Methods

Mouse skin sample collection, RNA extraction and Reverse transcription

All animal works were performed under the license of the University of Bradford (Bradford, UK) and the Institutional Animal Care and Use Committee protocol of Boston University (Boston, MA, USA). Skin samples were collected from neonatal C57BL/6 mice at postnatal days 12–23 (P12–P23), as well as from 8- to 10-wk-old adult mice. Skin was frozen in liquid nitrogen and embedded, as described elsewhere [34]. Chemically induced skin carcinogenesis mouse model has been previously described [34]. Mouse skin tissue collection at different stages of chemically induced skin carcinogenesis, total RNA extraction and nucleic acid quantity and quality analysis were performed as previously described by the manufacturers. Briefly, tissues were homogenized in a lysis buffer, and RNA was isolated using silica column-based purification, followed by on-column DNase treatment to remove contaminating genomic DNA. The quality and quantity of the RNA were assessed using a spectrophotometer and agarose gel electrophoresis. The cDNAs were synthesized using the same technique, as described below for the reference cDNA.

Reference cDNA Template Preparation (Reverse Transcription)

Reference cDNA template preparation was performed by using the two-step method. Total mouse embryonic RNA at E 19.5 was reverse transcribed to cDNA using the following cycling conditions in the thermal cycler:

Table 1 Cyclic conditions for cDNA synthesis

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25ºC	37ºC	85ºC	4ºC
Time	10 minutes	120 minutes	5 minutes	hold

High-Capacity cDNA reverse transcription kit 200 reactions without RNAse inhibitors and with random primers by Applied Biosystems was used to make the master mix. The master mix was prepared for 5 reactions and each reaction had 10µl of the 2X reverse transcription master mix and 10µl of the RNA sample (2µg/10µl). All preparations were done on ice and all solutions were centrifuged briefly during the experiment.
After the reverse transcription, the synthesized cDNA was amplified by regular PCR. Eppendorf licensed and authorized PCR machine was used and the amplification was performed by following the cyclic conditions:

Table 2 Cyclic conditions for cDNA amplification

Steps	Temperatures	Times	Number of cycles
Initial Denaturation	94ºC	3 minutes	1 cycle
Denaturation	94ºC	30 seconds	35 cycles
Annealing	58ºC	10 seconds	
Extension	72ºC	30 seconds	
Final Extension	72ºC	3 minutes	1 cycle
Soak	4ºC	indefinite	1 cycle

Reagents (Promega) with GAPDH reverse and forward primers were used to make master mix for 10 reactions. Taq polymerase was added last to the master mix, each component was mixed and centrifuged briefly and all preparations were done on ice. Each reaction had 24.5µl of the master mix and 0.5µl of the template. 3 different templates were used for 3 different reactions; water control, reference RNA control (1µg/µl) and the reference cDNA.

Annealing Temperature Optimization for the Real Time PCR

Annealing temperature optimization reaction was performed in BIO-RAD, MyiQ, Single Color Real Time PCR machine. Perfecta SYBR Green Supermix for IQ (VWR) was used as a Pre-formulated real-time PCR master mix. Commercially available software program Beacon Designer was used to perform both primer design and amplicon selection for SATB1 and SATB2 (Appendix I). Two different master mixes were prepared for SATB1 and SATB2 primer pairs with adding water and the reference cDNAs with the SYBR Green supermix. Each PCR plate well had 10µl of this master mix. After the loading, the plate was sealed with the BIO-RAD microseal® 'B' Film PCR sealer and centrifuged for 2 minutes at 4000rpm. qPCR reaction was run by using the iCycler iQ® system on the qPCR instrument and the temperatures B (65.9), C (63.8), D (60.8), E (56.5), and F (53.4) were used to find the annealing temperature.

Melting-curve analysis along with the Agarose gel electrophoresis was used to check the specificity of this real time PCR amplification reaction and to identify any additional non-specific products and Primer-dimers. 26 PCR cycles were chosen for melting-curve analysis because they are sufficient to amplify the target DNA to detectable levels without over-amplifying, which could lead to non-specific products or primer-dimers. This cycle number typically ensures that the reaction is in the exponential phase of amplification, where each cycle produces a measurable increase in target DNA. Therefore, 26 is a common choice for balancing amplification efficiency and specificity for melting-curve analysis. Each well on the agarose gel had 8µl of the samples from the PCR plate and 2µl of the loading buffer. 5µl of the molecular weight marker was used to identify the sizes of the DNA fragments in the gel.

Assay Performance Evaluation Using Dilution Series

2-Fold dilution series from the reference cDNA tube, which had 6µl of the cDNA and 54µl of water, were prepared to make the dilution factors of 1, ½, ¼, 1/8 and 1/16. Two different master mixes were prepared for SATB1 and SATB2 with the SYBR Green supermix, forward and reverse primers and water, similarly to the description above for annealing temperature optimization. Each master mixes were prepared for 12 reactions. Each well on the PCR plate had 2µl of the cDNA dilution and 8µl of the master mix. Duplicate samples were used in order to make a statistical analysis of the experiment and to perform a performance evaluation of the assay. A Control reaction also included to the PCR plate with adding water to the master mix. The determined annealing temperature from the optimization reaction above was used and the real time PCR was performed by following the cyclic conditions:

Table 3 Cyclic conditions for Real Time PCR

Steps	Temperatures	Times	Number of cycles
Initial Denaturation	95ºC	3 minutes	1 cycle
Denaturation	95ºC	10 seconds	40 cycles
Annealing	56.5ºC	30 seconds	
Extension	72ºC	15 seconds	

Melting curve analysis	65ºC - 95ºC	30 seconds	26 cycles

The standard curves were constructed by plotting the dilution factor against the threshold cycle (CT) value obtained during amplification of each dilution. Then, the linear regression lines were constructed by plotting the log2 of the dilution factors against the threshold cycle (CT) value obtained during amplification of each dilution. The equation of the linear regression line along with the coefficient of determination and the amplification efficiency equation were then used to evaluate whether the qPCR assay was optimized.

Gene Expression Analysis with the qRT-PCR

After the optimization of qPCR conditions by using the reference cDNA, and after the assay performance evaluation, the relative expression levels of SATB1 and SATB2 at different stages of the mouse skin carcinogenesis were measured by qRT-PCR. The experiment was performed twice in duplicates in order to determine if the data was reproducible. In the first experiment, three different conditions (three different cDNAs) were used: 12 days after the depilation (late anagen skin) as a control, early carcinogenesis (15 weeks after the initiation of carcinogenesis) and middle carcinogenesis (25 weeks after). In the second experiment, the 3 days cDNA after the depilation (early anagen skin) was also added as a second control to the above conditions. In this study, the GAPDH gene was used as housekeeping gene for normalization. SATB1, SATB2 and GAPDH primer pairs were used in each experiment and the master mixes were prepared for these each pair of primers as described previously. For experiments 1 and 2, the master mixes were prepared for 18 and 24 reactions (addition of the early anagen control) respectively. Each well had 8µl of the master mix and 2µl of the cDNA template. The same cyclic conditions of the dilution series experiment above were used. Depending on both practical and scientific considerations, the gene expression analysis was performed as a singleplex assay and DNA-binding dye SYBR Green 1 (BIO-RAD) was used.

Agarose Gel Electrophoresis

1.5% Agarose gel was prepared with TAE buffer and the products of the regular PCR amplification were run at 100volts for 40 minutes to observe the synthesized and amplified reference cDNAs. 6µl of the Invitrogen 1Kb plus DNA Ladder (50ng/1µl) was used as a molecular weight marker. Each well had 20µl of the sample and after 40 minutes, the gel was stained with Ethidium bromide (0.5µg/ml). The gel documentation system (Ingenius Syngene Bioimaging) was used to document and analyze the agarose gel image.

Data Analysis

The relative quantification was performed by using the reference gene GAPDH. After determining the CT values of both the target and the reference genes, the relative expression levels of the target genes in the test samples (in each condition) were determined by using the 2–ΔΔCT (Livak) Method.

All statistical analyses were performed using SPSS software program version 17.0 (PASW Statistics). Data are presented as mean and standard deviation. The relative expression levels between the conditions were statistically analyzed using an independent two-sample t-test that does not assume equal variances (with a significance level at the $p \leq 0.05$ level).

Results

Reference cDNA synthesis and amplification

First, the reference cDNA was synthesized from total mouse embryonic RNA at E 19.5 to optimize the qRT –PCR assay conditions for SATB1 and SATB2. The quality of cDNA was checked by regular PCR using GAPDH specific primers. The gel electrophoresis analysis demonstrated the presence of one specific product of expected size (128 bp) confirming the good quality of the cDNA. The clear single bands can be observed in lanes B to E on the gel image below (Figure 1). These results indicate that the reference cDNA samples can be used for the annealing temperature optimization of SATB1 and SATB2 and also for the dilution curve experiment.

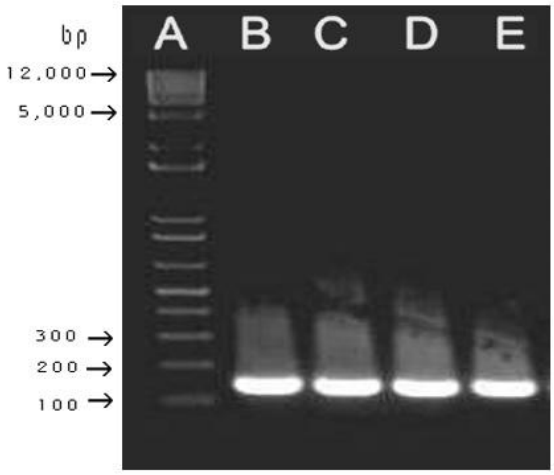


Fig 1 Reference cDNA synthesis and amplification. Lane A: Invitrogen 1Kb plus DNA Ladder, Lanes B to E: PCR products after the amplification of reference cDNA with GAPDH primers

Annealing Temperature Optimization for SATB1 and SATB2 qRT–PCR Assay

Annealing temperature was chosen as the major parameter for PCR optimization. Amplification with SATB1 and SATB2 primers was tested at five different annealing temperatures (0C): B (65.9), C (63.8), D (60.8), E (56.5), F (53.4). Table 1 below shows the Threshold Cycle (CT) values for the SATB1 and SATB2 primers at each temperature.

Table 4 CT values of SATB1 and SATB2 at all temperatures during the amplification of the optimization

Temperature (^{0}C)	Identifier	Threshold Cycle (Ct)	Set Point
B	SATB1	25.43	65.87
B	SATB2	N/A	65.87
C	SATB1	24.63	63.849
C	SATB2	36.56	63.849
D	SATB1	24.33	60.758
D	SATB2	23.56	60.758
E	SATB1	23.90	56.462
E	SATB2	22.86	56.462
F	SATB1	24.10	53.395
F	SATB2	22.92	53.395

These results indicate that the optimum annealing temperature is 56.50C (E) for both SATB1 and SATB2 primers. At the temperature 56.50C, SATB1 and SATB2 primers showed the lowest CT values indicating the highest amplification efficiency at this temperature. Appendix II contains the amplification graphs of the SATB1 and SATB2 at the temperature 56.5 and at all tested temperatures during the optimization process.

Melting-curve analysis along with the agarose gel electrophoresis was used to check the specificity of this real time PCR amplification reaction during the annealing temperature optimization.

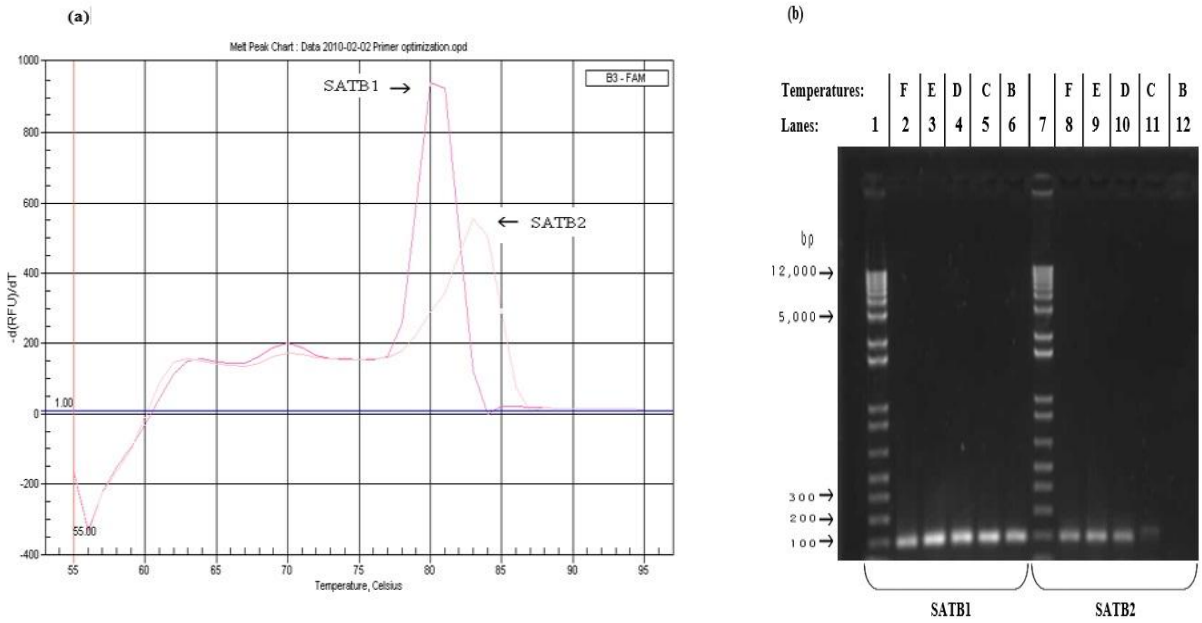


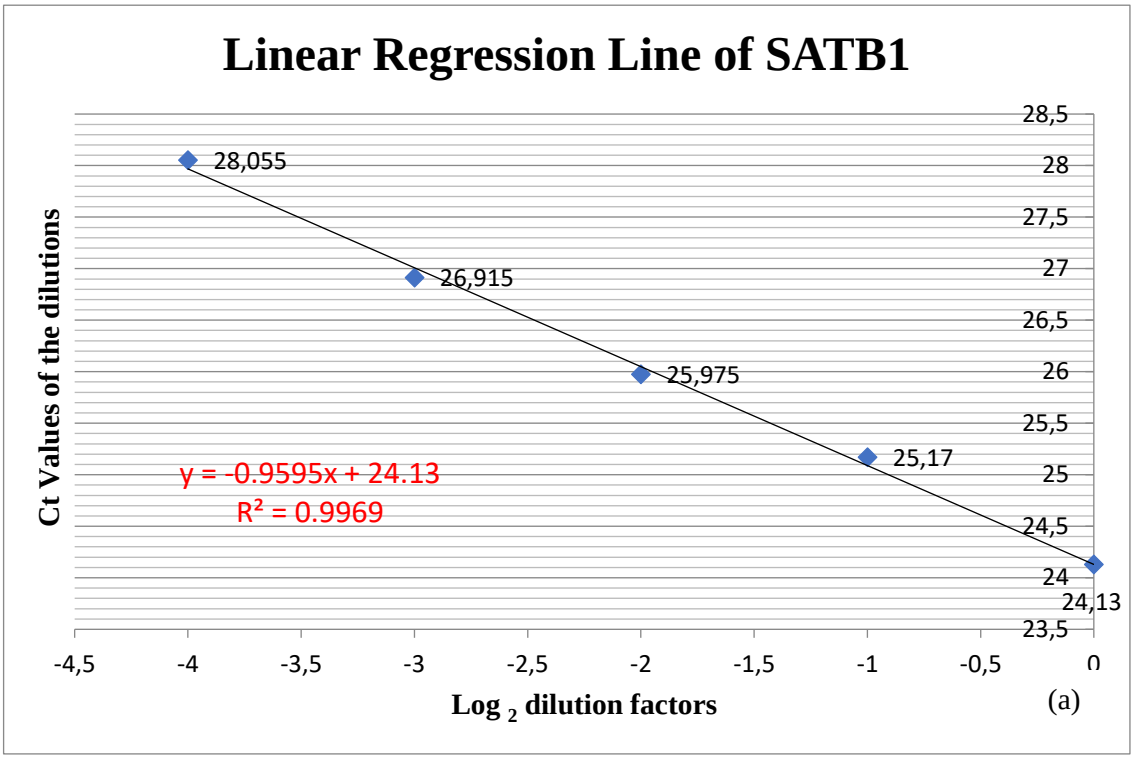
Fig 2 (a) Melting-curve analysis chart of SATB1 and SATB2 at 56.50C (Temperature E). (b) The Agarose Gel Electrophoresis of SATB1 and SATB2 PCR products at all temperatures with the molecular marker (Lane 1 and 7) Invitrogen 1Kb plus DNA Ladder (5µl/lane).

The melting-curve analysis of the SATB1 and SATB2 at the annealing temperature 56.60C has shown only one peak for each primer pairs demonstrating the high specify of the amplification. The agarose gel electrophoresis also confirmed the presence of the single specific product for each primer pair, of expected sizes (184 bp for SATB1 and 188 bp for SATB2). These results demonstrated that the melting curve analysis was consistent with the agarose gel electrophoresis and there were no contamination or non-specific products during the PCR amplification. The annealing temperature of 56.60C was chosen for SATB1 and SATB2 primer pairs based on maximal amplification efficiency and specificity.

Assay Performance Evaluation

2-Fold dilution series of the reference cDNA were made to prepare the mixtures with the dilution factors 1 (undiluted), 1/2, 1/4, 1/8 and 1/16. The amplification CT values were obtained in each dilution factor for SATB1 and SATB2 at the end of the real time PCR (Appendix III). The mean value of the duplicate samples at each dilution were calculated and the standard curves were constructed for SATB1 and SATB2 by plotting the dilution factor against the threshold cycle (CT) value obtained during amplification of each dilution (Appendix IV).

By using these standard curves, the linear regression lines were constructed, by plotting the log2 of the dilution factors against the threshold cycle (CT) value obtained during amplification of each dilution (Figure 3). After plotting the linear regression lines, the coefficient of determinations (R2), Slopes and amplification efficiencies were calculated (Appendix V). For SATB1 assay, the R2 was 0.9969 and the amplification efficiency was 94.6%. For SATB2 assay, R2 was 0.9984 and the amplification efficiency was 104.6%. The parameters of the dilution curves are in the range considered suitable for the SATB1 and SATB2 assays. These results indicate that the optimized assay conditions can be used to determine the SATB1 and SATB2 mRNA expression levels in the experimental samples.



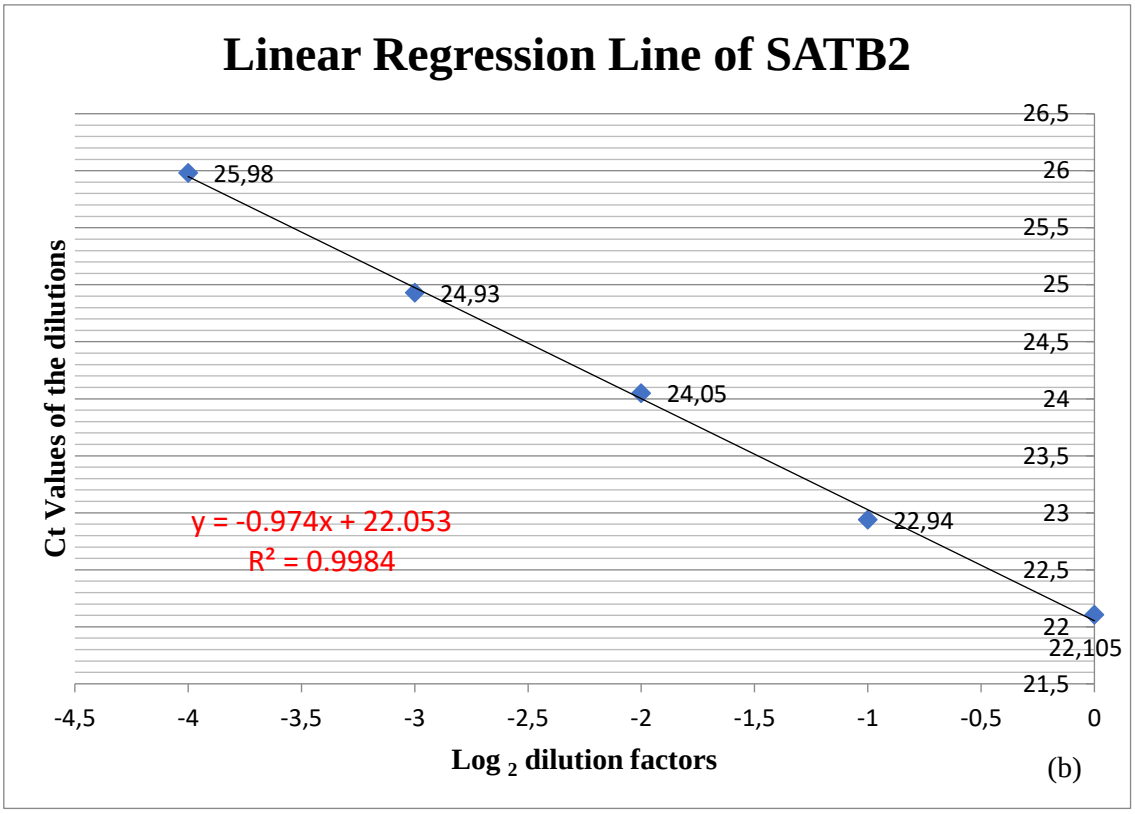


Fig 3 (a) Linear Regression Line, formula and R2 value of SATB1 (b) Linear Regression Line, formula and R2 value of SATB2.

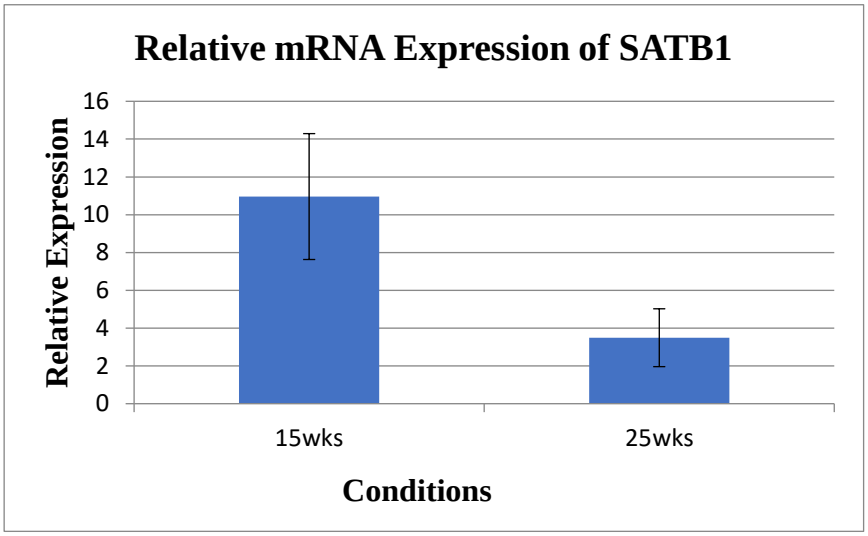
SATB1 and SATB2 Expression Analysis in Mouse Skin at Early and Middle Stages of Chemically Induced Carcinogenesis

The expression levels of SATB1 and SATB2 mRNAs were determined by quantitative RT-PCR. The experiment was performed twice in duplicates with GAPDH as the housekeeping gene and the amplification CT values were obtained from both experiments (Appendix VI). These CT values demonstrated that the cDNAs for the control conditions had poor quality. Therefore, the comparison between SATB1 and SATB2 expression levels at early (hyperproliferative epidermis) and middle (papillomas) stages of skin carcinogenesis was performed.

Figure 4 below was constructed by combining the results from both experiments. It shows the relative expression levels of the SATB1 and SATB2 mRNAs at early and middle stages of the skin carcinogenesis. The bars in Figure 4 represent the mean of duplicate samples from both experiments and the error bars were constructed by calculating the standard deviation of these samples.

These results demonstrated that in comparison to the early stage of skin carcinogenesis, both SATB1 and SATB2 mRNA expressions are lower in the middle stage of skin carcinogenesis. Comparing to SATB1, a stronger down-regulation of the SATB2 in the middle stage of skin carcinogenesis was also observed.

(a)



(b)

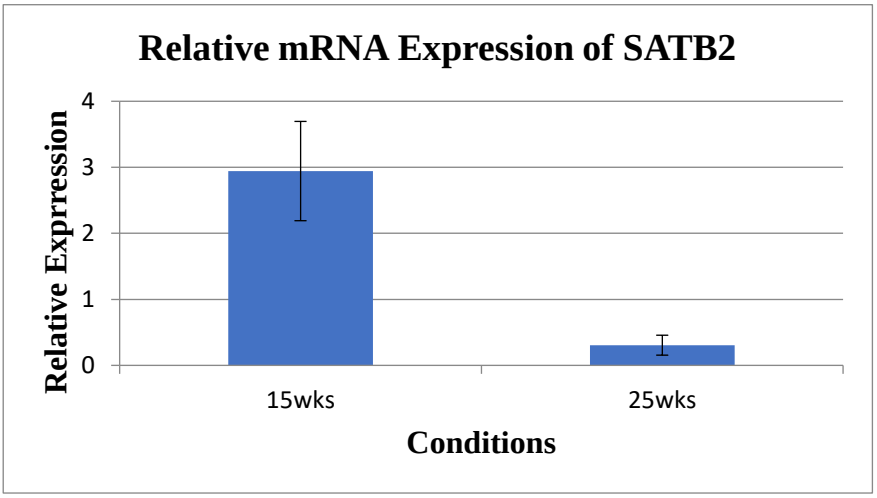


Fig 4 (a) The relative expression levels of SATB1 at early (15 wks) and middle (25 wks) stages of skin carcinogenesis. (b) The relative expression levels of SATB2 at early (15 wks) and middle (25 wks) stages of skin carcinogenesis.

The two-sample F-test for variances was used to test the equality of variances (Appendix VII). It was determined that the variances of the two groups in SATB1 expressions and in SATB2 expressions were significantly different, p=0.0288 and p=0.0002 respectively. Therefore, the relative expression levels between the experimental conditions were statistically analyzed using the independent two-sample t-test that does not assume equal variances.

The statistical analysis confirmed the significant downregulation of SATB1 ($p = 0.0001$) and SATB2 ($p = 1.05 \times 10^{-5}$) expression in papilloma-containing skin compared to hyperproliferative skin in the mouse model of chemically induced carcinogenesis (Appendix VIII).

These results show a significant downregulation of SATB1 and SATB2 mRNA expression in the middle stage of skin carcinogenesis compared to the early stage.

Conclusion and Discussion

The findings of the present study demonstrate the SATB1 and SATB2 expression levels in mouse skin at early and middle stages of chemically induced carcinogenesis. It was determined that there was a down-regulation of SATB1 and SATB2 during the middle stage of (papillomas) skin carcinogenesis. Results of the statistical analysis demonstrate a significant difference in the expression levels of SATB1 and SATB2 between the stages of early and middle skin carcinogenesis. The comparison of SATB1 to SATB2 in the expression levels has shown that SATB2 has a greater down-regulation in the middle stage of skin carcinogenesis.

In contrast to the present study, a previous report suggested high-expression levels of SATB1 in metastatic breast cancer cells and in multidrug-resistant breast carcinoma cell lines [26]. However, the findings of the present study are consistent with the previous report that indicates normal SATB1 expression levels in other many cancer cells [27]. In the present study, results obtained from measurements of SATB2 expression are consistent with the previous report that suggests a relationship between low-expression levels of SATB2 and tumor progression in colorectal cancer [17]. However, significantly higher levels of SATB2 mRNA expression were determined in breast cancer specimens and expression was associated with increase tumor grade [35].

Since the potential role of SATB1 and SATB2 gene activity in tumors of the skin is unknown, this study provides a fundamental knowledge about their association with skin cancer by measuring the expression levels during the different stages of the skin carcinogenesis. However, this study only demonstrates the expression levels of SATB1 and SATB2 at early and middle stages of skin carcinogenesis. Since the data for the expression levels of SATB1 and SATB2 in the normal skin cells is lacking, a confident comparison of SATB1 and SATB2 expression levels in mouse skin carcinogenesis to expression levels in normal mouse skin cells can not be stated. Although this result was not achieved, it can be speculated that both SATB1 and SATB2 were not found to be over-expressed during this study.

Due to the time and resource limitations, the late stage of skin carcinogenesis (carcinomas) wasn't added to the testing conditions. Future research should include appropriate controls such as early and late anagen skin samples along with a telogen skin sample and also the late stage of skin carcinogenesis and metastatic tumors conditions should be included to measure the expression levels at all different stages and make a more reliable conclusion and comparison. Also, the SATB1 and SATB2 gene expression analysis was performed using only the qRT-PCR method with SYBR Green Dye. Therefore, the analysis of the gene expression could be also performed with qRT-PCR with TaqMan probes to increase the specificity of the detection during the expression analysis. Other methods such as Northerring blotting or Tag based serial analysis of gene expression (SAGE) along with the qRT-PCR method could be also used for the gene expression analysis.

The future aspect of this study would be the extension in variety of the techniques used during the experiments of the present study. These techniques can be used to reveal the roles of SATB1 and SATB2 in normal and cancer mouse skin cells by studying their functions, structures, locations in the nucleus and their interactions with the transcriptional factors and chromatin modifying molecules. Such studies would provide a comprehensive knowledge about SATB1 and SATB2 genome-wide targets and about the various factors recruited by SATB1 and SATB2. This progress would further enhance our understanding of the intricate relationship between chromatin architecture and genome function [36].

There is growing evidence that the transcriptional activity of genes might be influenced by nuclear organization [37] and as mentioned before SATB1 has a structure that provides the ability to bind to AT-rich DNA sequences and interact with chromatin remodeling complexes. It functions as a genome organizer and

regulates gene expression by recruiting chromatin remodeling/modifying enzymes and transcription factors to genomic DNA, which it tethers via BURs. It has been shown that a variety of genes involved in many aspects of tumorigenesis are regulated by SATB1, indicating that a large group of SATB1-targeted genes collectively induce tumor growth and metastasis [26]. According to these findings, high expression levels of SATB1 can be associated with carcinogenesis by causing the accumulation and binding of these transcriptional factors and chromatin modifying enzymes to the targeted DNA sites.

Few studies have investigated the role of the SATB2 gene in skin carcinogenesis. The present study raises the intriguing possibility that the observed downregulation of SATB2 during the middle stage of skin carcinogenesis may contribute to tumor development, similar to its role in colorectal cancer (CRC), where its reduced expression correlates with tumor progression and poor prognosis [17]. In CRC, SATB2 downregulation has been linked to increased tumor invasion, lymph node metastasis, distant metastasis, and advanced Dukes' classification [17]. The stronger downregulation of SATB2 in skin carcinogenesis suggests that its loss may play a critical role in disrupting normal epidermal differentiation and promoting a more aggressive tumor phenotype. SATB2 is known to function as a chromatin organizer, regulating gene expression programs essential for cell identity and differentiation. A significant decrease in SATB2 expression may lead to widespread epigenetic changes that favor tumor progression by suppressing differentiation-associated genes while upregulating pathways involved in proliferation, invasion, and survival.

Moreover, given that chromatin remodeling is increasingly recognized as a key regulatory mechanism in cellular differentiation [38], the downregulation of both SATB1 and SATB2 in this study may reflect a broader disruption of differentiation pathways in skin carcinogenesis. A previous study suggested that SATB2 can inhibit SATB1's effects, indicating potential regulatory crosstalk between these two chromatin remodelers [17]. This raises the possibility that the loss of SATB2 may enhance SATB1-driven oncogenic programs or lead to an imbalance in gene expression networks critical for maintaining normal epithelial homeostasis.

Furthermore, SATB1 and SATB2 play crucial roles in regulating gene expression in embryonic stem cells, where their relative levels help balance self-renewal and differentiation [35]. Thus, the significant downregulation of SATB2 observed in this study may contribute to a shift toward a less differentiated, more tumorigenic state in skin carcinogenesis. Further investigations are needed to determine the precise molecular mechanisms through which SATB2 loss influences tumor progression and whether restoring its expression could have therapeutic potential in skin cancer.

Genetic studies in mice have identified multiple signaling pathways and transcriptional regulators that are essential for proper epidermal stratification, acquisition of the skin barrier function [6], maintenance, differentiation and lineage commitment of the epidermal stem cells (SCs) [7]. These pathways involve Notch, Wnt/β-catenin, Hedgehog, c-Myc, mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) and the transcriptional regulators p63 (which is related to p53), the AP2 family, the CCAAT/enhancer-binding protein (C/EBP) transcriptional regulators, interferon regulatory factor 6 (IRF6), grainy head-like 3 (GRHL3) and Kruppel-like factor 4 (KLF4) [39]. These signaling pathways regulate the functions of skin epithelial SCs, and deregulation of these signaling pathways leads to the development of cancer in various tissues [6]. An important question for the future is whether adult skin SCs are the only cells that sufficiently accumulate mutations to cause cancer and whether the SATB1 and SATB2 play an important role in this process. This hypothesis is attractive because adult SCs exist and proliferate for a long time. Also, they do not protect their genome by asymmetric chromosome segregation [40], providing further opportunities to accumulate oncogenic mutations and potentially induce cancer formation.

SATB1 and SATB2 have been extensively studied in various cancers, highlighting their roles in tumor progression and differentiation. As mentioned before, SATB1 is frequently overexpressed in breast and colorectal cancers, where it promotes metastasis, epithelial-mesenchymal transition (EMT), and tumor cell survival [41]. In lymphomas, SATB1 influences T-cell differentiation and proliferation [41]. Conversely, SATB2 often acts as a tumor suppressor in colorectal cancer but has oncogenic roles in osteosarcoma and certain breast cancer subtypes [42]. These findings suggest that SATB1 and SATB2 may contribute to skin carcinogenesis through similar mechanisms, such as chromatin remodeling, transcriptional regulation of differentiation pathways, and modulation of the tumor microenvironment. Further studies are needed to elucidate their precise roles in skin cancer progression and therapeutic resistance.

In the present study, the common chemical carcinogenesis regime (two-stage induction) was used to promote the skin carcinogenesis in the mouse model. Current information suggests that skin tumor promoters are not mutagenic but bring about a number of important epigenetic changes, such as epidermal hyperplasia and increase in polyamines, prostaglandins and dark basal keratinocytes [43]. The generality of the two-stage

system of inducing tumors has been shown to exist in a number of experimental carcinogenesis systems, such as liver, lung, colon, stomach, bladder and pancreas. However, it is not presently known if other experimental carcinogenesis systems and the induction of human cancer involve a series of stages similar to that in the mouse skin [43].

One subject that remains to be explored is how SATB1 and SATB2 expression levels are associated with the tumor growth and metastasis in various tissues including the skin. Despite the recent interests to SATB1 and SATB2 gene activity, the structural and functional properties of these special AT-rich sequence-binding proteins and their possible roles in various cancer cells are still not fully understood. Since the association of these proteins with cancer is a relatively new field of study, further studies of the biological functions of SATB1 and SATB2 are needed to understand the mechanisms of occurrence and development of various cancers including skin cancer.

Abbreviations

SATB1: Special AT-rich sequence-binding protein 1, SATB2: Special AT-rich sequence-binding protein 2

RT-PCR: Reverse Transcription Polymerase Chain Reaction, SCs: Stem Cells, SP: Side-population, NM: Nuclear Matrix, MARs: Matrix Attachment Regions, BURs: Base-unpairing Regions, CRC: Colorectal Cancer, qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction, CT: Threshold Cycle

DMBA: 7,12-dimethylbenz[a]anthracene, TPA: 12-O-tetradecanoylphorbol-13-acetate, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, SPSS: Statistical Package for the Social Sciences

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Data Availability statement

The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards

Conflict of interest

The author declares no conflict of interest.

Ethical standards

The study is proper with ethical standards.

Authors' contributions

During the study, VY conducted the research and wrote the article.

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