Epigenetic Regulation of the Tumour Suppressor RASSF1A in Bone Cancer Cells: DNA Methylation Study

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ABSTRACT

Objective: Osteosarcoma is a bone cancer that affects children and adolescents. The RASSF1A is a tumor suppressor capable of mediating the regulation of cell cycle arrest, migration, including apoptosis. It is the most continually silenced gene that contributes to human cancer. Furthermore, RASSF1A functions as a scaffold protein that can regulate microtubules network and bind apoptotic kinases MST1 and MST2 via the Sav-RASSF-Hippo domain. Epigenetic inactivation of genes by DNA methylation is a key factor regulating gene expression and genomic stability. Our aim was to study the RASSF1A gene promoter methylation in three osteosarcomas (U2OS, Saos-2, and MG-63), two Ewing Sarcoma (A-673 and SK-ES-1), and one fibrosarcoma (HT-1080) cell lines.

Materials and Methods: Three osteosarcomas (U2OS, Saos-2, and MG-63), two Ewing Sarcoma (A-673 and SK-ES-1), and one fibrosarcoma (HT-1080) cell lines were used to study RASSF1A gene promoter methylation, using bisulphite conversion of DNA, followed by methylation-specific polymerase chain reaction (PCR).

Results: The RASSF1A’s gene promoter methylation was established as a frequent event. Hypermethylation of RASSF1A promoter, was detected in five out of six studied cell lines.

Conclusions: These results demonstrated that altering the Sav-RASSF1-Hippo may be accomplished through hypermethylation of RASSF1A and may play an essential role in Ewing’s sarcoma and Osteosarcoma. The methylation pattern of Sav-RASSF1-Hippo tumor suppressor pathway in human bone cancer along with RASSF1A expression with its effector proteins merits further investigation. This may reveal how the RASSF1A has a physiological signal transduction, including how the process of its deregulation can contribute to transformation of the cell, eventually leading to the incorporation of novel therapeutic options with improved prognosis for bone cancer.

Keywords: RASSF1A, Osteosarcoma, DNA Methylation, Epigenetics

INTRODUCTION

A primary malignant tumor of the bone known as Osteosarcoma, results in formation of immature bone tissue by tumor cells. According to the World Health Organization, bone tumors are classified into central and surface tumors (1). Almost 90% of all osteosarcoma cases are represented with central high-grade primary Osteosarcoma, which is the third most common type of neoplasia after leukemia and lymphoma, among children and adolescents (2). Treatment mainly involves conducting standard chemotherapy before surgery and after completion and then subjecting it to radiation with a 5-year survival rate of 60–70%. However, the survival rate of patients with locally advanced or metastatic tumors remains low (~20%), with the median survival time of only 23 months (3). Evidence from osteosarcoma studies suggests that Osteosarcoma could be a result of failure in the differentiation program of the cells of origin mesenchymal stem cells (4). Moreover, chromosomal instability (CIN) is a hallmark of Osteosarcoma, which represents a high level of genomic instability (5). However, the mechanisms underlining osteosarcoma metastasis are still unidentified. Thus advances in identifying biomarkers for osteosarcoma metastasis and therapeutic regimens are urgently required.
Both genetic and epigenetic events control cancer initiation and progression. Epigenetics is defined as altering gene expression while maintaining the DNA sequence (6). The epigenetic aberrations are reversible, which is a great potential to develop epigenetic therapies to treat cancer (7). The most important epigenetic mechanisms are MicroRNA, histone modifications, and DNA methylation (8, 9). DNA methylation is defined as the covalent addition of a methyl group to the nitrogenous base cytosine at CpG dinucleotides on the DNA strand (10, 11). Methylation of CpG dinucleotides result in inactive promoter which associate with a “closed” chromatin structure, inaccessible by the transcription factors (12). Altered methylation pattern leads to genomic instability, which result in the initiating of tumorigenesis and persistence in the malignant state of cancer cells (7, 13). DNA methylation represses the tumor suppressor’s gene expression and therefore removes regulatory proteins required for normal cell growth and development and thus causing cancer (14, 15).

Evidently, RASSF1A is a compelling tumor suppressor, and considered as one of the most perturbed genes that contributes to human cancers. Interestingly, RASSF1A functions as an upstream regulator of Hippo pathway, importantly, the Hippo/mammalian STE20-like protein kinase MST1and MST2 pathway has a connection with its tumour suppressor activity. Though, the Hippo pathway comprises of a large network of kinases cascade that control cell proliferation and cell death (16). The kinases consisting of MST1 and MST2 cascade include large tumor suppressor LATS1 as well as LATS2, including the adaptor proteins Salvador homologue 1 (SAV1) (16). Activation of MST1 and MST2 results in phosphorylation of LATS1, LATS2 and downstream cell cycle regulation and apoptosis (17). RASSF proteins are critical in the regulation of the MST1 and MST2 by preventing forming a complex with a protein called RAF1, and cleavage of this inhibitory protein is actively regulated by RASSF1A which activates the hippo pathway (18). Sav1 interacts with MST1/2 forming a complex that phosphorylates the LATS proteins. When phosphorylated, LATS1 and LATS2 activate Yap1 protein which leads to its nuclear relocalization and binding to p73 and induces apoptosis (Figure 1) (18, 19).

It is worth noting that, RASSF1A is a tumor suppressor gene that assumes the role of upstream regulator of Hippo pathway initiating kinase cascade that phosphorylates and negatively regulates transcription by transcriptional coactivators. Hyper methylation of RASSF1A results in the loss of function of the Hippo pathway which triggers Osteosarcoma. When RASSF1A restores the MST2 and interaction with LATS1 leads to phosphorylation of LATS1 by MST2, resulting to Yap1 phosphorylation as well as its nuclear translocation therefore, inhibiting Yap1 oncogenic function. Eventually, Yap1-p73 complex is formed and induce apoptosis, when the pathway is activated by RASSF1A.

The process of CpG methylation islands, associated with the promotor regions of RASSF1A, results in reduced expression of the functional protein and loss of the tumor suppressor activity (20, 21, 22, 23). The role of the hippo pathway in cancer has been extensively studied, with events such as mutations and methylation of the promoters present in many different cancers and correlating with poor prognosis (24). To our knowledge, with regard to primary bone tumors, limited studies from the past to current publications have synthesized the presence of changes in methylation for several genes, combining RASSF genes. Due to the fact that methylation changes frequently appear during the early stages of the disease, the detection of hypermethylated genes may help in the identification of tissues derived from patients with increased risk.

The aim of this study is to identify the prevalence of RASSF1A promoter hypermethylation that may alter (Sav-RASSF-Hippo) pathways, thus contributing to the cancer phenotype. Investigating the potential activation of the Sav-RASSF1A-Hippo tumor suppressor pathway at the pathogenesis of bone cancer involved the analysis of hypermethylation of RASSF1A in bone cancer cell lines. The process involved the bisulphite modification of DNA and then the methylation-specific PCR.

**MATERIAL and METHODS**

**Cell culture**

All the six cell lines namely, U-2-OS, A-673, Saos-2, SK-EK-1, HT-1080 and MG-63 were assessed and purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All media for cell cultures were acquired from ATCC, whereas supplements such as fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco (Gaithersburg, MD, USA). The brief characteristics of cell lines and protocol for cell culture and maintenance are given below.

**Cell lines**

**U-2 OS:** Cell line U-2 OS is altered in chromosomal way, having chromosome counts in the hypertriploid range. This cell line was obtained from a moderately differentiated sarcoma of the tibia of a girl aged 15 years. For culturing, vials were softened or melted by introducing a gentle agitation at a temperature of 37°C water bath and decontaminate using a spray containing 70% of ethanol. The content of vial was then kept in a centrifuge tube containing 9.0 ml of an entire culture medium and was spun at approximately 125xg for 7 minutes. The cell pellet was suspended in component of McCoy’s 5a Medium (ATCC) having 10% FBS (Gibco) and transferred into a culture flask calibrated at 75 cm2. The cells were kept warm at 37°C using an incubator containing 5% CO₂.

**A-673:** The A673 cell line was obtained from a patient that had a probability of primary rhabdomyosarcoma. Such kinds of cells have a history of producing several growth factors with oncogenic potential, including cell growth-inhibitory factors. Hence the cell lines were made to grow using the Dulbecco’s Modified Eagle’s Medium containing 10% FBS.

**Saos-2:** Saos-2 is derived from the primary Osteosarcoma of an 11-year-old Caucasian girl. For culturing, cells were subjected to growth using McCoy’s 5a Medium that was made of 15% FBS.

**SK-ES-1:** SK-ES-1 is a human Ewing sarcoma (anaplastic Osteosarcoma) cell line that displays epithelial morphology and grows in adherent. This cell line was established in 1971 from a bone biopsy in an 18-year-old Caucasian male with...
Ewing’s sarcoma. For culturing, cells were subjected to the process of growth in McCoy’s 5a Medium having 15% FBS.

**HT-1080:** The cell line is derived from tissue taken in a biopsy of a fibrosarcoma present in a 35-year-old human male. Briefly, vials were softened gently at a temperature of 37°C water bath and decontaminate and then sprayed using 70% ethanol. The contents of vials were then poured into a centrifuge tube containing 9.0 ml of complete culture medium and were spun at approximately 125xg for 7 minutes. The cell pellet was suspended in Eagle’s Minimum Essential Medium (EMEM) containing 10% FBS and poured into culture flask with a capacity of 75 cm². The cells were kept warm at a temperature of 37 °C and by using an incubator containing 5% CO₂.

**MG-63:** MG-63 cell line is derived 14-year-old Caucasian male suffering with an osteosarcoma. These cells were grown in EMEM containing 10% heat-inactivated FBS.

**Protocol for the Process of Subculturing**

The process began by removing the culture medium followed by rinsing of the flask using 0.25% -EDTA solution (0.03%). Subsequently, an additional 2 ml of trypsin-EDTA solution was added and the flask was maintained at a temperature of 37°C until the cells detached. This followed by an addition of fresh culture medium and the cells and dispensing of the cells into new culture flask with subcultivation ratio of 1:4.

**Freezing cells**

Back-up cells were made using Cryopreservation for future studies. The medium was separated from flask; the cells were cleaned and trypsinized as well as using trypsin-EDTA solution. Once cells are detached, 5-10 ml media was added to the flask and the contents are transferred in to a 15 ml centrifuge tube. The cells were pelleted by spinning down at 1500 rpm for approximately 5 minutes and then followed by removal of the medium. Cells were then re-suspended in enough in a medium of a freezing state (Complete culture medium + 5% DMSO), creating a cell suspension and aliquot of about 1ml were made into storage vials. Cells were immediately transferred to -20°C for one hour, followed by -80°C overnight before permanent storage in liquid nitrogen.

**DNA extraction**

The cells were harvested and were transferred into 1.5ml centrifuge tubes. The Genomic DNA was obtained using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Briefly, according to the manufacturer instruction, the cells were lysed using nuclei lysis buffer, and RNase digestion step was included at this time. The cellular proteins are then removed by a salt precipitation step, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation. The DNA concentration and purity (260/280) were checked using Nano-drop spectrophotometer.

**Bisulfite conversion**

Bisulfite conversion of DNA is possible due to the presence of bisulfite-mediated and chemically conversion of unmethylated cytosine residues into uracil.

Evidently, there was no change in Methylated cytosine residues. Sequence-specific PCR primers can distinguish unmethylated genomic regions after bisulfite conversion (24).

The other approach involved subjecting Aliquots of 2 μg of each DNA sample in bisulfite modification by employing the Methyl Edge Bisulfite Conversion System (Promega) following the manufacturer instructions. The next stage involved a brief mixing of 2 μg of DNA with 130 μl containing bisulfite conversion reagent. Then, there was bisulfite conversion using an effective thermal cycling program: denaturation at 98°C for 8 minutes followed by another warm maintenance at 54°C for 60 minutes. After re-using, the bisulfite-converted DNA purification on a spin column and eluted in a volume of 20 μl and stored at -20°C.

**Methylation analysis**

At this level, methylation-specific PCR (MS-PCR) was used in the analysis of the methylation status of supporter areas of RASSFIA. One hundred nanograms of bisulfate preserved DNA were PCR improved in 20 μl reaction buffer containing 10x Taq hot start master mix (New England Biolabs, Ipswich, MA).

Prime sequences for Methylation-specific primer forward (MSP-F): CGAGAGCGGTTTGGTGTTCGT;

Methylation-specific primer reverse (MSP-R): CGATTAACCGGTACTCGCTAA;

Unmethylated specific forward (USP-F): GGGGTTTTGGAGACGTTGTT;

Unmethylated specific reverse (USP-R): CCAATTAACCCCATCTCACCTAA.

PCR reactions for all the genes were performed using Touchdown (TD) PCR method. TD-PCR provides modest and fast methods to enhance PCRs, aggregating specificity, compassion, and yield, without extending the length and design of the primers. Any alteration in Tm between the right and wrong annealing will result in an exponential benefit of the twofold per cycle (25). The cycling program for TD-PCR comprises two discrete stages. Stage 1 is the landing point that consists of ten cycles with a galvanizing temperature above the melting temperature (Tm) of the primers being used and changes to a less annealing temperature over the course of consecutive cycles. Phase 2 is a generic intensification period of 20 or 25 cycles using the last galvanizing temperature stretched in Phase 1. Phase 1 annealing temperature of 63 °C to 53 °C for both MSP & USP primers, for 10 cycles followed by phase 2 with annealing temperature of 53 °C for 30 cycles. The extension was performed at 68 °C for 30 sec. PCR products were unglued on 2.5% Agarose gel and envisioned with ethidium bromide.

The findings of abnormal supporter methylation of the RASSFIA gene in all six studied cell lines have been recurrent six times to promote the accuracy of the results.
RESULTS

The results of aberrant promoter methylation of RASSF1A gene in all six studied cell lines have been repeated six times to ensure robust outcomes.

Methylation Analysis of RASSF1A

Genomic DNA of cell lines was treated with bisulfite alteration to illuminate the position of RASSF1A hypermethylation in sarcoma cell lines. Thereafter, we analyzed the status of RASSF1A promoter CpG Island methylation by MS-PCR. The PCR was conducted using two diverse mechanisms and galvanizing temperatures to prevent the probability of a non-specific PCR product. The use of a relatively great difference (50 °C) in Tm of the primers utilized, descent PCR procedure (highest galvanizing temperature 63 °C and lowest at 53 °C) was also tracked to prevent the occurrence of non-specific PCR products.

Two cell lines (lines (SK-ES-1 and HT-1080) from the six investigated were thoroughly methylated while three cell lines (U-2-OS, A-673 and Saos-2) were incompletely methylated and no cell line was left unmethylated. However, as demonstrated by (Figure 2) no methylation was found with MG-63.

MS-PCR was used to analyze the methylation position of the RASSF1A promoter area in sarcoma cell lines. MSP uses both Methylation-specific (m) and unmethylation-specific (u) primers, which were set on 2.5% agarose gels with the addition of a marker (100 bp). The expected PCR product for MSP is 192 bp and for USP is 204 bp. Touchdown PCR, annealing temperature (53 °C). N denotes normal DNA from leucocytes.

Figure 1. Schematic diagram of RASSF1A protein signal and modulation of hipo pathway components. It is worth noting that, RASSF1A is a tumor suppressor gene that assumes the role of upstream regulator of Hippo pathway initiating kinase cascade that phosphorylates and negatively regulates transcription by transcriptional coactivators. Hyper methylation of RASSF1A results in the loss of function of the Hippo pathway which triggers Osteosarcoma. When RASSF1A restores the MST2 and interaction with LATS1 leads to phosphorylation of LATS1 by MST2, resulting to YAP1 phosphorylation as well as its nuclear translocation therefore, inhibiting YAP1 oncogenic function. Eventually, YAP1-p73 complex is formed and induce apoptosis, when the pathway is activated by RASSF1A.

Figure 2. MS-PCR of the RASSF1A CpG Island. MS-PCR was used to analyze the methylation position of the RASSF1A promoter area in sarcoma cell lines. MSP uses both Methylation-specific (m) and unmethylation-specific (u) primers, which were set on 2.5% agarose gels with the addition of a marker (100 bp). The expected PCR product for MSP is 192 bp and for USP is 204 bp. Touchdown PCR, annealing temperature (53 °C). N denotes normal DNA from leucocytes.
DISCUSSION

Osteosarcoma is a malevolent tumor of the bone mostly found in children. Although there has been tremendous therapy to increase the rate of cancer survival, it is still the same place it was decades ago. One of the basic inactivation methods is silencing tumor suppressor genetic factors by epigenetic alteration. It reduces the impacts of cancer-related genetic factors in the pathogenesis of human tumors. Hypermethylation plays a significant function in suppressing tumor genes (26).

According to Jones and Baylin, RASSF1A is a vital tumor reducer gene that is involved in trials controlling cell cycle seizure and apoptosis. RASSF1A inactivation has been identified in various malignancies (26). Moreover, it has been inactivating at various high frequencies to produce positive outcomes. Aberrant methylation of RASSF1A was often perceived in many cancers. As demonstrated by Li et al., (27), RASSF1A methylation is found in 80% of minor cell lung tumors, in 90% of hepatocellular carcinomas, and more than 70% of prostate tumors. Moreover, RASSF1A was revealed to be a cancer suppressor in Osteosarcoma and presented to be methylated in Ewing sarcoma. The results of the study conquer with the previous report as they also show that RASSF1A was absolutely methylated in 2 Ewings sarcoma (SK-ES-1 and A-673) cell lines (28). Lim et al., also carried out a study on the manifestation of RASSF1A in key osteosarcomas and cell lines. The authors demonstrated a lack of RASSF1A manifestation in 4/10 key and 5/6 cell lines (29). Moreover, a recent study by Malpeli et al. showed that the methylation of two CpG islands situated at RASSF1A promoter controls its manifestation. Although physiological suppression of RASSF1A manifestation by methylation remains unknown, it is believed to be through cell governing procedures (30). RASSF1A promoter hypermethylation leads to the alteration of its composite governing network that is essential for tumor growth (31). Moreover, studies have found that the hypermethylation of the RASSF1A promoter causes the transcription of its merging isoform RASSF1C that may bind to RASSF1A downstream effector (32, 33). This splicing also avoids the regulation of diverse biological purposes, including cell cycle seizure, apoptosis, migration, control of the microtubule network, and autophagy (34, 35). Demethylation agent (5-aza-2'-deoxyctydine) was used to treat RASSF1A-negative cell lines and RASSF1A manifestation was upregulated, signifying that RASSF1A promoter methylation can be a probable strategy for the transcriptional suppression of RASSF1A (36, 37). The current study also established that the RASSF1A is methylated in 2/3 bone cancer cell lines (U2OS and Saos-2) to reduce the development of cancer. In contrarily, Harada et al. (38) demonstrated that the two cell lines (SAOS-2 and A-673) did not present methylation of the RASSF1A. Therefore, we had to repeat the experiment more than three times due to this contradiction. The experiments were conducted more than two times to prevent false results and conclusions. However, we found that the results were reproducible. Our results are supported by the work of Lim et al. (29) because they also found that a relationship between osteosarcoma and RASSF1A hypermethylation. Moreover, Dammann et al. also found that RASSF1A hypermethylation suppressed cancer genes. They demonstrated that out of eight of the pancreatic carcinoma, five cell lines (PATU-S, PATU-T, PATU-2, PaCa-2, and CAPAN2) were fully methylated, while two cell lines (HUP-T3 and HUP-T4) were partly methylated and only CAPAN1 was unmethylated (39).

Hippo is a preserved governing trail, first recognized in drosophila, and is significant in controlling organ growth, cell propagation, and cell loss. Conspicuously, the hippo/MST trail has been statistically related to the cancer suppressor action of RASSF1A. According to Aruna et al., primary elements that have been studied include LATS1, LATS2, MST1, MST2, and Sav1. Furthermore, DNA methylation was found to diminish MST1 appearance in glioblastoma and sarcoma (40). MST1 and MST 2 are the mammalian orthologues of the drosophila hippo protein and an existing primary element in the hippo trail. Aruna et al., (40) found that RASSF1 interrelates through its C-terminal domain with MST1 and thus controls MST1 arbitrated apoptosis. Nevertheless, it is not yet determined whether a similar approach exists in Osteosarcoma. Recent evidence revealed that RASSF1 is participating in the crossroad of a compound governing network, which includes key regulators of cellular homeostasis, for instance, MST2/Hippo, Ras, p53, and death receptor pathways. Moreover altered methylation and expression of RASSF1A is one of the greatest actions in solid cancers (41).

Accruing proof backs the utilization of RASSF1A hypermethylation as a prognostic biomarker that relates with deprived prognosis, and demonstrates that its inactivation has a primary task in tumor growth. For instance, studies investigating neck, head, and renal cancers revealed that cancers with high levels of RASSF1A methylation not only have a negative outcome but development to metastatic illness significantly earlier than other cancers (42, 43, 44). Therefore, the initiation of RASSF1A is an effective mechanism for treating numerous cancer types.

CONCLUSIONS

RASSFs suppress protuberant cancer and control microtubule steadiness, apoptosis, and cell cycle. Although the specific process of the RASSF biological task is complex, comprehending it is highly beneficial because it may lead to the discovery of effective anticancer drugs. The results of the study demonstrate epigenetic dormancy of RASSF1A through methylation was evident in Osteosarcoma, fibrosarcoma, and Ewing’s sarcoma. RASSF1A gene methylation analysis can serve as the foundation of cancer diagnostic tests. Classifying different methylated CpG investigations between the osteosarcoma cell lines helps identify novel epigenetic biomarkers that can probably provide optional methods of recognizing cancer subtypes. Future studies on methylation data analysis will determine whether there exists a correlation between tumor stages, metastasize, and survival rate.

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