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Histones: Controlling Tumor Signaling Circuitry

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Abstract

Epigenetic modifications constitute the next frontier in tumor biology research. Post-translation modification of histones dynamically influences gene expression independent of alterations to the DNA sequence. These mechanisms are often mediated by histone linkers or by proteins associated with the recruitment of DNA-binding proteins, HDAC I and II interacting proteins and transcriptional activators, coactivators or corepressors. Early evidence suggested that histones and their modifiers are involved in sophisticated processes that modulate tumor behavior and cellular phenotype. In this review, we discuss how recent discoveries about chromatin modifications, particularly histone acetylation, are shaping our knowledge of cell biology and our understanding of the molecular circuitry governing tumor progression and consider whether recent insights may extend to novel therapeutic approaches. Furthermore, we discuss the latest oncogenomic findings in Head and Neck Squamous Cell Carcinoma (HNSCC) from studies using Next Generation Sequencing (NGS) technology and highlight the impact of mutations identified in histones and their modifiers.

Keywords

H3; H4; Oral cancer; Whole genome sequencing; Genetic code; Histone modifications; Acetylation; Chromatin compaction

Introduction

With the completion of the human genome it becomes evident the upcoming opportunities to explore and identify new genome-scale mechanisms involved in gene expression. The field of epigenetics presents a unique opportunity to understand the molecular mechanisms underlying the modulation of gene expression in the absence of alterations to the DNA sequence. Epigenetics is involved in the control of normal tissue homeostasis through maintenance of tissue-specific genetic signatures. However, deregulated environmental cues

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can corroborate to cellular transformation by altering local signaling circuitry, resulting in dysfunctional epigenetic equilibrium. By interacting with environmental cues, tumors undergo genetic deviations resulting in changes on cancer behavior, cellular phenotype, increase aggressiveness or induction of tumor dormancy. Therefore, it is important to understand the crosstalk between environmental signals and tumor behavior in epigenetics.

Oncogenomics, which uses high-throughput technologies to screen cancer-associated genes, is a novel strategy for tackling the complex interaction between DNA mutations and epigenetic driven deviations. DNA mutations originate from changes in the nucleotide sequence mainly derived from insertions, deletions, point mutations and chromosomal translocations that influence the normal balance between cellular proliferation and death. In an effort to identify new biomarkers and develop novel cancer therapeutics, multiple mega oncogenomic projects were initiated. These included the Cancer Genome Project at the Wellcome Trust Sanger Institute and the Cancer Genome Anatomy Project (CGAP), a National Cancer Institute initiative aimed at determining the genetic expression of normal, precancerous, and cancerous cells.

In the last several years, oncogenomics applications have extended from identifying genes associated with cancer to analyzing transcript abundance in tumors (Transcriptome) [1,2], DNA methylation (Methylome) and histone acetylation (Acetylome) [3,4]. The transcriptome is highly influenced by histone modifications that are primarily driven by acetylation and methylation. Histone modifications directly influence chromatin DNA packaging, resulting in a decondensed structure and enhanced interactions with transcription factors. This process, referred as the histone code, confers a high degree of cellular plasticity in both normal cells, such as the neuron synapse and lymphocytes [5,6], and tumor cells of the ovary and head and neck [7–9]. We highlight below how histones and its modifications are involved in stem cell biology and in the molecular circuitry governing tumor progression. We also present and discuss the involvement of histone acetyltransferase, histone deacetylases and its inhibitors as a therapeutic strategy used in various cancers. Further, we explore recent findings on head and neck cancer mutations of histones and histone modifiers identified using next-generation sequencing.

The Chromatin: Epigenetic Switches of the Genome

The genome is organized and packed into the nucleus through interactions with core histone proteins. Differential DNA folding regulates access to regulatory elements, resulting in alterations of the cellular response to environmental cues. DNA folding modifies cellular phenotypes by inducing cell type-specific chromatin organization [10]. We recently reported on functional chromatin folding in head and neck squamous cell carcinoma (HNSCC) cell lines during tumor response to environmental cues that dynamically modulates tumor behavior and cellular phenotype [9].

The ability of the chromatin to modulate cellular behavior is associated with how tightly DNA is spooled around H2A, H2B, H3 and H4 core histones [11]. Together, histones and DNA form nucleosomes, the fundamental units of chromatin. Each nucleosome is composed of 146bp of DNA coiled in sequence around a core of eight histone proteins [12].

Histones, the most abundant proteins associated with DNA, were highly conserved during evolution and are associated with the regulation of nuclear gene expression in several tissue types. Histones maintain contact with DNA through their flexible globular domain and amino acid residue regions (lysine, serine and arginine) that protrude from the nucleosome (histone “tail” domain). The majority of posttranslational modifications in histones, such as lysine acetylation, serine phosphorylation, lysine methylation, arginine methylation, and lysine ubiquitination, occur at the residue region [13–17]. The pattern of histone modifications determines chromatin status (euchromatin or heterochromatin) [18], the accessibility of DNA to nuclear factors and ultimately transcription. Euchromatin is relatively uncondensed and represents loci being actively transcribed. In contrast, heterochromatin is condensed and represents areas of the genome that are silenced. Several studies show that alterations in chromatin structure due to histone modifications correlate with gene expression, the cell cycle, DNA replication and damage, DNA repair and chromosome stability [19,20].

Dynamic changes in chromatin structure organization are mainly driven by histone acetylation and deacetylation, mechanisms responsible for important regulatory functions of gene expression. These processes are controlled by histone acetyltransferases (HAT) and histone deacetylases (HDACs), enzymes that are targeted to specific amino acid positions. In histone acetylation, HATs catalyze the addition of an acetyl group to the lysine residue in the tails of histones resulting in neutralization of the positive charge in the histone tail. The new acetyl group reduces the histone-DNA interaction, resulting in open or active chromatin that allows binding of transcription factors to promoters of target genes. Therefore, increased levels of histone acetylation are generally associated with increased transcriptional activity [21]. Acetyl groups are removed in histone deacetylation, resulting in increased histone-DNA interaction and chromatin condensation that blocks transcription by preventing the binding of transcription factors and regulatory complexes [21–23].

HATs and HDACs act in a coordinated manner to influence gene expression in different tissues. These enzymes induce conformational changes in histone-DNA interactions that alter the accessibility of transcription factors to DNA and are crucial epigenetic changes for the development and differentiation of many normal cell types. However, altered HAT and HDAC activity has also been observed in several types of cancer and may be promising therapeutic targets for these diseases [13,21,24].

Epigenetic regulation of gene expression is primarily controlled by histone acetylation and methylation, histone modifications, and changes in nucleosome conformation [20]. There is growing evidence that epigenetics is involved in gene regulation during mammalian development (reviewed in [25]). These processes are observed during the maintenance of embryonic stem cells (ES) in the early stages of embryogenesis. Polycomb group (PcG)-protein repressive system is an example of epigenetic control of gene expression. PcG form a multiprotein complex involved in chromatin organization and capable of epigenetically induce modifications on its structure thereby influencing gene transcription. In ES cells, PcG repressive system suppresses developmental pathways, thereby maintaining ES cell pluripotency and plasticity during embryonic development [26]. Furthermore, Boyer *et al*. identified several PcG target genes in ES cells bound by OCT4, SOX2 and NANOG, three

transcription factors associated with pluripotency [27]. Similarly, the inability of the PcG protein repressive complex to suppress developmental genes in ES cells results in spontaneous cellular differentiation [26]. In accordance with the PcG-protein repressive complex, the effect of HDAC on chromatin organization regulates the maintenance of stem cell pluripotency in coordination with numerous signaling pathways [28,29]. In contrast, chromatin condensation in tumors is associated with chemoresistance [30–33] through the reactivation of stem cell-like transcription programs [34]. In addition to the known functions of the PcG-protein repressive complex, recent reports have shown that the histone variant H2A.Z plays a similar role in early development. H2A.Z assists with the dissociation of H2A–H2B dimers, resulting in modifications to the nucleosome surface that promotes transcription factor binding [35]. H2A.Z induces changes in chromatin configuration allowing access to transcription factors and cofactors that activate and repress regions in embryonic stem cells leading to self-renewal and differentiation [36,37]. This mechanism involves the depletion of nucleosomes at the Transcription Start Sites (TSSs) where gene transcription begins. Recently, Schones *et al*. found that nucleosomes containing H2A.Z are particularly susceptible to depletion [38]. Further, Li *et al.* observed by high-resolution nucleosome mapping and chromatin immunoprecipitation sequencing (ChIP-Seq) that co-occupancy of the transcriptional activator FoxA2 and H2A.Z results in nucleosome depletion during ES cell differentiation, coinciding with gene activation [37].

Histone Acetyltransferase (HAT) and Histone Deacetylases (HDAC)

The equilibrium between histone acetylation and deacetylation in the promoter region of chromatin is essential for the regulation of gene expression and the establishment of a transcriptionally competent chromatin state [39–42]. The antagonistic effects of HDACs and HATs tightly control the balance of steady-state acetylation. While HDACs promote transcriptional repression by removing acetyl groups from lysine, HATs increase transcriptional activity through the addition of acetyl groups. Besides these electrostatic changes, HATs and HDACs also play roles in gene activation or silencing through the recruitment of co-activators and co-repressors of transcription.

Alterations in histone locus-specific post-translational modifications are proposed for describing a code, which defines the state transcriptional potential of a cell referred as the code of histones. A global comprehension of this code is necessary for dissecting the role of histones in physiology and pathology [43]. Initial steps towards this goal have been made with the characterization of the histone deacetylase-1 enzyme (HDAC1) as a key regulator of embryonic stem cells proliferation and repression of cell cycle inhibitors in mice indicating its requirement during embryonic development [44]. Impairment of HAT activity or overexpression of HDAC by aberrant gene expression is associated with the process of carcinogenesis and tumor progression [13,21,45,46]. Because histone modifications do not alter the DNA sequence, new agents have been developed that reverse this modification, resulting in reactivation of tumor suppressor genes and reestablishment of a signaling balance to maintain cellular homeostasis. Notably, inhibitors of histone deacetylases (HDACi) are a new group of anticancer agents that have shown promising results [47].

Histone acetyltransferase (HAT) and its Inhibitors

The HATs are divided into the following five distinct families based on sequence divergence in the HAT domain: (i) HAT1, (ii) Gcn5/PCAF, (iii) MYST, (iv). CBP/p300, and (v). Rtt109. HAT activities involve protein complexes and co-activators of transcription factors [48]. Various biological processes are associated with HAT function, including cell cycle progression, DNA damage repair, dosage compensation and hormone signaling [24].

The HAT family possesses diverse catalytic strategies for acetyl transfer, reflecting its long evolutionary time. Several HAT substrate peptides, including histone H3 (centered around Lysine 14) and histone H4 (centered around Lysine 8), have been described [24]. HATs have substrates beyond histones, such as non-histones proteins and transcription factors. Certain substrates are transcriptional cofactors for a range of cellular oncoproteins, such as MYB, JUN, FOS, RUNX, BRCA1, p53, and pRB, and viral oncoproteins, including E1A, E6, and SV40 large T [13, 24, 49, 50]. Of note, p53 was the first functional non-histone substrate identified to be acetylated by HATs [51] reviewed in [52]. P53 is acetylated by CBP/p300 at multiple lysine residues (positions 370, 372, 373, 381, and 382) in the C-terminal regulatory domain, which result in increased DNA binding activity and acetylation-induced conformational changes, and protein stabilization [53–55].

Translocation, amplification, overexpression or mutation of HAT genes are found in a variety of human pathologies, including cancer, inflammatory lung disease, viral infection, diabetes, fungal infection, and drug addiction [48,56–58]. The CBP/p300 HAT genes are considered functional tumor suppressors because they reside in regions frequently lost in tumors, and certain cancers exhibit mutations that abrogate p300 activity [59–61]. For example, the tumor suppressor function of CBP/p300 was confirmed in animal models with ablated *Cbp* [62,63]. Similarly, translocation partners, including MLL, MOZ, and MORF [64–66], disrupt CBP/p300 in leukemia. These findings established a new paradigm for transcriptional regulation and tumorigenesis by showing that epigenetic regulation of genes collaborates with genetic alterations in cancer development [18,51, 58,67].

Clinical trial assessment of HAT inhibitors is in the early stages, especially compared to the multitude of trials testing kinase inhibitors. There is also interest in natural compounds, such as anacardic acid and garcinol, that inhibit hPCAF and hp300, respectively. Other natural compounds like Curcumin, Epigallocatechin-3-gallate (EGCG) and plumbagin have been shown to selectively inhibit CBP/p300. New HAT inhibitors have been developed that display submicromolar inhibition activity and selectively inhibit Gcn5/PCAF and CBP/p300 [24,58,68]. There is great interest in developing additional HAT inhibitors with improved specificity and anti-tumor activity.

Histone deacetylases (HDACs) and HDAC inhibitors

HDACs are cellular enzymes expressed in many tissues that play important roles in differentiation, proliferation, and cancer. HDACs are primarily involved in the acetylation network, and several HDACs have been described in humans. Based on similarities, HDACs are divided into four functional classes based on sequence homology to their corresponding transcriptional regulator of yeast counterparts (*Saccharomyces cerevisiae*): 1) class I

(HDAC1, HDAC2, HDAC3 and HDAC8); 2) class II is subdivided into class IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and class IIb (HDAC6 and HDAC10); 3) class III (SIRT1 to SIRT7); and 4) class IV (HDAC11 and related enzymes) [13,39,69,70]. These enzymes are part of multi-protein complexes, and their activity depends on other factors, such as mSin3, N-CoR and SMART transcriptional co-repressors. Changes in these factors can indirectly interfere with HDAC activities [21].

HDACs also have numerous non-histone target proteins comprised of transcriptional regulators (Rb, DEK, HMGI(Y)/HMGA1 and others), transcription factors (p53, c-Myc, BCL-6, E2F1 and others), DNA repair enzymes (Ku70, TDG, NEIL2 and others), nuclear import regulators (Rch1 and importin-α7), chaperone proteins (HSP90), structural proteins (α-tubulin), viral proteins (E1A and others) and inflammatory mediators (HMGB1) [71]. These proteins are involved in cellular proliferation, differentiation and cell death, and their functions can be intrinsically modulated by protein acetylation [23,71].

Class I HDACs are ubiquitously expressed in all tissue types [72,73]. Of particular interest for this review, HDAC1 and HDAC2 are involved in proliferation, regulation of the cell cycle and apoptosis. Posttranslational modifications of HDAC1 and HDAC2 alter their enzymatic activity, subcellular localization, stability, and interaction with other binding sites in response to different stimuli. In addition, deregulation of HDAC1 and HDAC2 is associated with the onset of many diseases in humans, including cancer [73]. HDAC1 suppresses activity of the p21WAF/SIP1 (cyclin-dependent kinase inhibitor) pathway by deacetylating lysine 4 residues on histone H3 [73,74]. Acetylation of HDAC1 dramatically reduces its enzymatic and repressive functions in vitro and in vivo, and overexpression of HDAC1 has been observed in many epithelial malignant tumors, such as hepatocellular [75], colorectal, gastric [76], pancreatic [77], and prostate [32] carcinomas. In contrast, depletion of HDAC1 results in perturbation of the cell cycle, loss of mitotic cells and increased cell death in several human cancer cell lines [78]. HDAC2 is overexpressed during the polyp stage of familial adenomatous polyposis colon cancer [79]. High levels of HDAC2 are also found in cervical dysplasia and invasive carcinomas [77]. Increased expression of HDAC1, HDAC2 and HDAC3 is associated with advanced stage prostate, gastric and colorectal cancers [76].

SIRT1, a member of the class III HDACs, acts as an important regulatory route capable of influence the apoptotic machinery. SIRT1 represses DNA damage-induced apoptosis through deacetylation of p53 [80]. SIRT1 also interferes with activation of apoptosis by deacetylating FOXO3a and/or E2F1 to inhibit their transcriptional activity [81,82]. Along with the control over the apoptotic machinery, SIRT1 promotes tumorigenesis by inhibiting cellular senescence, impairing cellular differentiation and promoting cellular growth associated with angiogenesis. SIRT1 is found highly expressed in many cancers, including acute myeloid leukemia, prostate carcinoma, and colon carcinoma, among others [83]. Further, SIRT1 is known to induce tumor resistance to chemotherapy suggesting its role as an oncogene (review in [87]). In contrast, SIRT1 is also considered a tumor suppressor gene due to its role in genomic stability, in limiting the replicative life span, in regulate cellular response to genotoxic stress and in the suppression of intestinal tumorigenesis [84–86].

Together, contradictory reports on SIRT1 function strongly suggests distinct roles depending on the involved signaling machinery.

HDACs inhibitors (HDACi) constitute a novel and growing family of potent anticancer agents that activate or repress 4–12% of genes by promoting histone acetylation, relaxing DNA that is coiled around core histones and increasing access of DNA to transcription factors [48,88–92]. In cancer cells, HDACi impair survival pathways resulting in cellular differentiation mediated by cell cycle arrest, block angiogenesis, induce apoptosis by stimulating the death receptor and intrinsic apoptotic pathway, and prevent tumor cells from evading the immune system by enhancing host immunosurveillance antigenicity (reviewed in [88,92,93]). Similar to SIRT1, the mechanisms involved in HDACi function are complex, differ between tumor cell types, and depend on the drug type and exposure time [92,94]. In vitro studies reveal the effects of HDACi are different for each type of cell lineage. However, common among cell types, is the activation of p53 and p21WAF1 and downregulation of cyclins and cell cycle activity following HDACi administration [95].

HDACi vary structurally and are classified in four groups, including hydroxamic acid, benzamides, cyclic peptides and aliphatic acids [90,92,96]. The potential of HDACi to exert anti-cancer effects has been examined in several clinical trials; however, only two HDACi agents have been approved by the Food and Drug Administration to date. SAHA (Vorinostat) and Romidepsin (Istodax) are approved for use in cutaneous T cell lymphoma (CTCL) [96–99]. Current clinical trials are examining HDACi in hematologic malignancies and solid tumors where preliminary results indicate a weaker antitumoral activity of HDACi in solid tumors compared to hematological malignancies [92,96,100]. However, many clinical trials involving solid tumors are comprised of patients with advanced disease and with relapsed and metastatic sarcomas (Table 1) (http://www.cancer.gov/clinicaltrials).

The most promising HDACi results involve its synergistic or additive effects with other therapeutic regimens, including radiotherapy, chemotherapy, proteasome inhibitors, death receptor agonists and kinase inhibitors [92,96,100,101]. This is evidenced in HNSCC, where *in vitro* administration of HDACi exhibits good anticancer properties; however, HDACi shows only limited clinical utility as a single agent [99,102–105]. HDACi is only effective against HNSCC when administered in combination with radiotherapy [106]. For example, Romidepsin shows preclinical activity as a radiosensitizer [101,107].

Although promising, the potential use of HDACi as a new treatment for HNSCC remains poorly explored. Kim *et al*. [108] examined the pharmacological efficiency of combining the PS-341proteasome inhibitor, also known as Bortezomib, with Trichostatin A, an HDAC inhibitor. They found the drugs synergistically induce apoptosis in HNSCC by a mechanism that preserves PS-341-induced endoplasmic reticulum stress. Erlich *et al*. analyzed the antitumor effects of AKT and PI3K-mTOR inhibitors delivered as monotherapies, or in combination with HDACi, in models of HNSCC. As a single agent, Vorinostat (HDACi) induces selective cytotoxicity in squamous cell carcinoma, an effect enhanced by PI3K inhibitors that cause persistent AKT inhibition and generation of reactive oxygen species [91]. Further, the use of Panobinostat, a non-selective HDACi resulted in a dose-dependent hyperacetylation of histone H3 and inhibition of tumor proliferation and viability when

administered alone. Further, the cytotoxicity effect observed upon administration of Panobinostat was continuously enhanced by co-treatment with PI3K inhibitors. Clinically, the administration of PI3K inhibitors enhanced the cancer cell specific cytotoxicity induced by Panobinostat. There are currently only four registered clinical trials testing HDACi and others therapeutic modalities in HNSCC. These studies involve Valproic acid and platinum-based chemoradiation in advanced HNSCC, Vorinostat in the treatment of advanced staged oropharyngeal squamous cell carcinoma, Capecitabine and Vorinostat in patients with recurrent and/or metastatic HNSCC and CUDC-101 plus cisplatin and radiation in HNSCC (Table 2) (http://www.cancer.gov/clinicaltrials).

Although the use of HDACi has generated great enthusiasm, adverse side effects and resistance have been observed in clinical trials of different cancer types. The main side effects are related to hematologic complications, but nausea, asthenia/fatigue, infections, vomiting, anorexia, diarrhea and pyrexia have been noted. Along with the side effects, the development of tumor resistance to HDACi have been noted although the involved mechanism is far from understood. Additional studies are needed to better understand the mechanisms underlying HDACi action, to explore various combination therapies and to identify which types of cancer will better respond to HDACi treatment [71,100].

The Histone Code of Chromatin Acetylation

Posttranslational chromatin modifications occur through changes to histones. Histones can be modified by acetylation, methylation, and ubiquitination of lysines, methylation of arginines, phosphorylation of serines, sumoylation and ubiquitination, among other recently identified covalent histone modifications [109]. The histone code hypothesizes that histone modifications dynamically influence transcription by recruiting a variety of proteins that activate transcription. Among the numerous histone modifications, we are particularly interested in the effects of chromatin acetylation on tumor behavior. Core histones are composed of a histone-fold domain and an amino-terminal tail domain. Histone acetylation is mediated through interactions of the amino-terminal domain with regulatory proteins that reduce the affinity of the histone to DNA [110]. Therefore, binding efficiency of transcription factors is directly associated with histone mediated changes in DNA configuration induced by acetylation [111,112].

We are just beginning to elucidate the effects of chromatin acetylation on cellular function. Initial observations by Meegee *et al*. [113] demonstrated the requirement of lysines to be acetylated in the histone 4 tail domain for cell cycle progression and genomic stability. Further, the acetylation status of histone 4 lysine 16 is implicated in numerous physiological functions across several organisms. In humans, loss of monoacetylation of histone 4 lysine 16 serve as an epigenetic marker for leukemia, breast, lung and colon cancer cells [114] and may be a key event associated with epigenetic silencing of tumor suppressor genes (TSGs) [115]. Interestingly, other histone 4 acetylation residues, such as lysine 5, 8, and 12, are not found in tumors, suggesting that suppression of histone 4 lysine 16 acetylation may be important for cancer prevention. Global patterning of histone acetylation is also observed during the development of the nervous system. Cho *et al*. demonstrated dynamic histone acetylation expression during neuronal differentiation, as evidenced by increased expression

of histone 3 and histone 4 in the cortical plate from rat cerebral cortex (E17) and chick spinal cord (E7) and increased histone 3 in adult mouse dentate gyrus [116]. In the cardiac development system, the HATs p300 and CBP are coactivators that promote the physiological and pathological growth of cardiac myocytes. Similarly, lungs and small intestines require p300 expression during normal development [117]. Collectively, these data suggest a role for acetylated histones in mitosis and differentiation during tissue development and maintenance. In fact, the identification of 3,600 lysine acetylation sites using quantitative mass spectrometry (MS)-based proteomics in 1,750 human proteins indicates that protein modifications mediated by acetylation is far more common than anticipated [118,119]. Although epigenetic modifications result in changes on gene expression independent from alterations on the DNA sequence, tumor epigenome may be influenced by heritable alterations on histones and histones associated transcriptional regulators. Indeed, next-generation sequencing of HNSCC has unveiled several of these mutations suggesting the differential modulation of epigenetic among normal and cancer cells and with that the existence of a cancer histone code. However, the functional and biological impact of these mutations in HNSCC is largely unknown.

The Next Generation of DNA Sequencing in HNSCC

Recent technological advances in the field of genome sequencing culminated in the development of Next-Generation Sequencing (NGS). This new technology has substantially improved discovery of single-base changes and the identification of larger structural variants characterized by insertions, deletions, translocations and viral insertions. Several sequencing platforms in use are characterized by amplification of single strands of fragmented DNA followed by sequencing [120].

The work of Agrawal *et al*. and Stransky *et al*. comprise the first HNSCC tumor sequencing studies using NGS technology and have provided a novel perspective for this complex disease. This wealth of data derives from 106 sequenced tumors from the head and neck anatomical area, including the oral cavity, oropharynx, hypopharynx, larynx, and sinonasal cavity. Stransky *et al*. have identified mutations of well-known genes, including *TP53*, which is present in 62% of sequenced tumors, and the *CDKN2A* gene, which occurs in 12% of tumors. In addition to *TP53* and *CDKN2A*, more novel gene mutations were also identified. For example, nonsense, missense and splice site mutations in Notch1 were found in 11% of 74 tumor samples analyzed [121]. Using NGS in 32 head and neck samples, Agrawal *et al*. found *NOTCH1* highly mutated in HNSCC, and approximately 40% of the mutations result in loss of function [122]. Both NGS studies found an inherited genomic difference between HPV-positive and HPV-negative head and neck tumors, with HPV-positive tumors containing less than half the number of mutations evident in HPV-negative tumors [121,122]. This suggests that HPV-positive HNSCC is genetically distinct from HPV-negative HNSCCs, a notion previously implied by their differential responses to chemotherapy and radiotherapy [123–125].

Novel NGS findings on HNSCC histones Mutations and its Modifiers

Within the thousands of genes found mutated in HNSCC, NGS have also identified mutations in histones and histones modifiers (Table 3). Of the 74 human head and neck tumor samples sequenced by Stransky *et al*. 44 tumors (59.5%) have mutated histones and/or transcriptional coactivators (Figure 1A). NGS analysis also revealed that 12 HNSCC have three or more mutations, and as many as eight mutations were found in a single tumor; 32 tumors harbor one or two mutations in histones and histone associated transcriptional regulators (Figure 1B). The most prevalent mutations are associated with genes encoding the H2A, H2B, H3, and H4 histone families. Similar mutations have been reported in the *H3F3A* and *HIST1H3B* genes in pediatric diffuse glioma [126,127], in *HIST1H4I* in clear cell renal cell carcinoma [128], and in *HIST1H4E* in human colon and rectal cancer [129]. Interestingly, mutations were also found in *HIST1H1B, HIST1H1C, HIST1H1D* and *HIST1H1E* linker histones. Linker histones are characterized by the absence of the histone fold domain and play a key role in organizing maximal nucleosome compaction within the chromatin [130,131] and chromatin binding affinity [132,133], among other functions. Notably, depletion of histone H1 induces aberrant mitotic figures [134], a common histological finding in tumor cells.

The Histone Lysine Demethylases (KDMs) are the second major family of genes mutated in HNSCC. KDMs, a new class of epigenetic enzymes, remove histone marks associated with gene expression and suppression. KDMs play a major role in prostate cancer by suppressing or promoting tumorigenesis [135].

NGS of HNSCC identified additional interesting gene mutations involved in transcription regulation. BCOR, and the BCORL1 encoded corepressor proteins are two examples of transcription regulators capable of selectively inhibit gene expression by interacting with the sequence specific DNA-binding protein BCL6. BCOR interacts with class I and II HDACs and may play an important role in repression. Both BCOR and BCORL1 are found in bone sarcomas and in acute myeloid leukemia [136,137]. The RCOR1 gene also found mutated in HNSCC encodes a corepressor protein that binds to the repressor element-1 silencing transcription factor (REST), but little is known about its involvement in tumor progression.

Transcriptional activators and coactivators are also mutated in HNSCC. Stransky's NGS identified mutations in *CREBBP* and *EP300*, genes that encode the well-known CREB-binding protein and histone acetyltransferase p300 (p300 HAT) transcriptional activators. CREB-binding protein and histone acetyltransferase p300 coactivators have inherent histone acetyltransferase activity and stabilize proteins at the transcription complex. Mutations in *CREBBP* and *EP300* are associated with the development of Rubinstein-Taybi syndrome and are involved in the development of acute myeloid leukemia. *EP300* is mutated in several epithelial tumors, including gastric, breast, cervical, colorectal, pancreatic, and gastric carcinomas [59,60,138–140]. The MYST family of histone acetyltransferases is composed of five human HATs: *Tip60* (*KAT5*), *MOZ* (*MYST3*), *MORF* (*MYST4*), *HBO1* (*MYST2*) and *MOF* (*MYST1*). *MYST1, MYST2*, and *MYST3* are mutated in HNSCC and were recently linked to cancer (reviewed in [141]). A putative role for *MYST1* is in modulation of ATM and acetylation of multiple residues of histone 4 [142]. *MYST2* is also involved in

acetylation of histone 4, but it plays a larger role in DNA replication and is a common site for retroviral integration [143]. *MYST2* occurs in a complex with the ING4 and ING5 tumor suppressors, suggesting it plays a suppressive role in carcinogenesis [144]. Similar to *MYST2*, *MYST3* forms a complex with ING5, suggesting that mutations in *MYST3* alter the ability of ING5 to regulate p53 function [145]. Lastly, mutation in the *EHMT1* histone methyltransferase suggests that transcriptional repression is a potential mechanism involved in tumor progression. The protein encoded by *EHMT1* is responsible for directly methylating lysine 9 of histone H3 and is responsible for Kleefstra syndrome (9q34 deletion syndrome) [146].

Conclusions

Increased knowledge of the biology of cancer is reflected in steady improvements in the overall survival rate of various cancers and the continuous reduction in cancer related death in the United States since the 1990s. The latest report on clinical advances in cancer therapy from the American Society of Clinical Oncology (ASCO) highlights the use of multitargeted drugs as the most efficient approach to manage different types of cancer. Emerging therapeutic strategies are using different drugs to target multiple components of common molecular pathways and have shown a positive impact on overall survival in patients with Gastrointestinal Stromal Tumors (GIST), metastatic colorectal carcinomas, neuroblastoma, Anaplastic Large-Cell Lymphoma (ALCL), medullary thyroid carcinoma, breast cancer, and metastatic sarcomas (http://www.cancerprogress. net). Unfortunately, the overall survival rate of HNSCC, one of the most common malignancies worldwide [147], has only marginally improved in the last 50 years despite significant efforts in identify new prognostic markers and understand the molecular signatures involved in tumor progression [148]. Until recently, the molecular circuitry associated with tumor invasion, metastasis and the development of chemoresistance was attributed to genetic instability driven by the accumulation of mutations. Recently, a better understanding of stem cells and developmental biology, in addition to technological advances in the field of full genome sequencing, are providing strong evidence that epigenetics equally contributes to cancer development.

Understanding the link between cancer mutations and epigenetic modulation of transcription will allow design of new therapeutic strategies that may improve the overall survival rate of HNSCC and other cancers. Advances in oncogenomics have also identified mutations in epigenetic associated genes that encode histones and their linkers, proteins associated with the recruitment of DNA-binding proteins, HDAC I and II interacting proteins, corepressor proteins and transcriptional activators and coactivators. Such mutations can dynamically confer tumor advantage over the transcriptional control of pro-survival pathways supporting tumor growth. The complexity of these interactions is evident in the differential effects of HDACi in in vitro models compared to the unpredictable outcomes observed in the clinic. Important questions remain to be answered including how mutations in histones and its modifiers influence tumor response to chemotherapeutic agents; how epigenetic signals presenting in the tumor microenvironment modulates tumor behavior; and how mutated epigenetic modifiers are capable of deregulate the balance between active and repressed transcriptional regions of the chromatin. Thus, understanding which gene mutations drive

histone modifications of enhancer and promoter regions may help us understand tumor susceptibility to HAT and HDAC inhibitors and guide the development new combination therapies.

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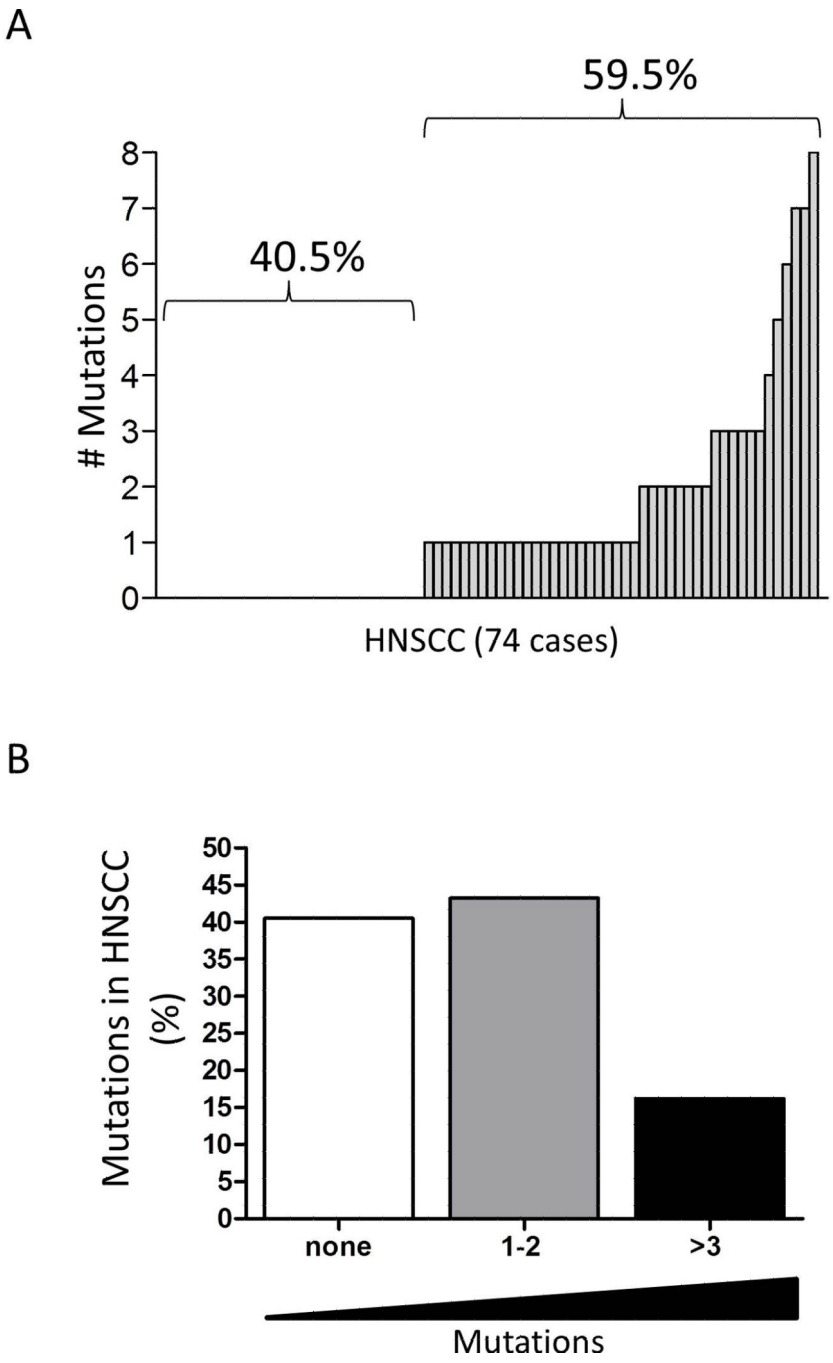

Figure 1. HNSCC mutations in histones and histone associated transcription regulators

(A) Bar graph of 74 sequenced HNSCC tumor samples [121] containing mutations in epigenetic associated genes that encode histones and their linkers, proteins associated with the recruitment of DNA-binding proteins, HDAC I and II interacting proteins, corepressor proteins and transcriptional activators and coactivators. (B) Bar graph depicting the percentage of mutations found in HNSCC samples (n=74). (Graph generated from the analysis of the HNSCC NGS supporting data published by Stransky *et. al.* 2011.

Table 1

Summary of Main HDACi Drugs and Agents in Clinical Trials.

Class	Compound	HDAC target	Phase	Company	Condition tested in Clinical Trials
Hydroxamic acid	Zolinza, vorinostat (SAHA)	Classes I and II	FDA	Merck	Approved for CTCL, phase I/ II for gastric cancer, NSCLC, thyroid cancer and advanced cancers
	Panobinostat (LBH589)	Classes I and II	III	Novartis	Phase III trial for CTCL and phase II for CML, MM, NHL, HL, BC, chordoma, prostate and others advanced solid tumors
	Belinostat (PXD101)	Classes I and II	II	TopoTarget	Relapsed ovarian cancer, TCL, thymoma, thymic carcinoma, MM, NSCLC, brain tumors
	Givinostat or Gavinostat (ITF2357)	Classes I and II	II	Italfarmaco	Relapsed leukemias and MM
	Trichostatin (TSA)	Classes I and II	N/A	N/A	N/A
	Abexinostat (PCI-24781)	Classes I and II	II	Pharmacyclics	BCL, sarcoma
	LAQ-824	Classes I and II	I	Novartis	Solid and hematologic malignancies
	Pracinostat (SB939)	Classes I, II and SIRT 1	II	MEI Pharma	Prostate cancer, solid tumors, hematologic malignancies
	JNJ-26481585	HDAC1, HDAC6	I	Johnson & Johnson	Leukemia, CTCL and solid tumors
	CUDC-101	Classes I and II	I	Curis	Head and neck, gastric, BC, liver cancer, MM, NSCLC
	CHR-3996	Class I	I	Chroma Therapeutics	Solid tumors
	CHR-2845	Class I	I	Chroma Therapeutics	Hematological diseases or lymphoid malignancies
	Quisinostat (JNJ 26481585)	Class I	II	MedKoo bioscience	CTCL, MM
	CG200745	Pan-HDAC	I	CrystalGenomics, Inc,	Solid tumors
	Rocilinostat (ACY-1215)	HDAC6	I and II	MedKoo bioscience	MM
Benzamides	Entinostat (MS-275 SNDX275)	HDAC1,HDAC2,HDAC3	II	Schering	HL, lung cancer, ALL, BC, melanoma and advanced solid tumors
	Mocetinostat (MGCD0103)	HDAC1 and HDAC2	II	Methylgene	AML
	Tacedinaline (CI-994)	HDAC1 and HDAC2	II and III	Parke-Davis Pharmaceutical	MM, pancreatic cancer, phase III for Lung Cancer
	AR-42	Pan-HDAC	I	Arno Therapeutics	MM, CLL or lymphoma
	4SC-202	HDAC1, HDAC2, HDAC3	I	MedKoo bioscience	Hematological malignancies
	Resminostat (4SC-201)	Classes I and II	I and II	MedKoo bioscience	Hepatocellular carcinoma, colorectal cancer and HL
Cyclic Peptides	Romidepsin/Istodax (FK228 and FR901228)	HDAC1 and HDAC2	FDA	Celgene	Aproved for CTCL

Class	Compound	HDAC target	Phase	Company	Condition tested in Clinical Trials
Aliphatic Acids	Valproic Acid	Classes I and IIa	II and III	Abbot	Phase III trials for cervical cancer, ovarian cancer and phase II for sarcomas, BC, malignant melanoma, AML, brain tumors and advanced cancers
	Phenylbutyrate (PB)	Classes I and IIa	I and II	Targon Corporation	Colorectal cancer, malignant gliomas, solid tumors, leukemias, lymphomas
	Butyrate	Classes I and IIa	I, II and III		Lymphoproliferative disorders, prostate cancer or other solid tumors, phase III for ovarian cancer

ALL: acute lymphoblastic leukemia; AML: Acute Myeloid leukemia; BC: Breast Cancer; BCL: B-Cell Lymphoma; CLL: Chronic Lymphocytic Leukemia; CML: Chronic Myeloid Leukemia; CTCL: Cutaneous T Cell Lymphoma; HL: Hodgkin Lymphoma; MM: Multiple Myeloma; N/A: not available; NHL: Non-Hodgkin Lymphoma; NSCLC: Non-Small- Cell Lung Carcinoma; TCLT: T Cell Lymphoma.

http://www.cancer.gov/clinicaltrials

Table 2

Clinical trials involving HDACi and head and neck squamous cell carcinoma.

Intervention Drugs	Condition	Sponsor	Patients enrolled	Phase	Period	Clinical trial number
Vorinostat (SAHA), Cisplatin, Radiation	Stage III or IV of SCC of the oropharynx	Ohio University Comprehensive Cancer Center	38	I	2010 2013	NCT01064921
Capecitabine (Xeloda), Vorinostat (SAHA)	Recurrent and/or metastatic head and neck cancer, Nasopharyngeal carcinoma, stage III and IV HNSSC and verrucous carcinoma	National (NCI) Cancer Institute	1445	II	2010 2013	NCT01267240
CUDC-101 Cisplatin, Radiation	Head and neck cancer	Curis, Inc.	22	I	2011 2013	NCT01384799
Valproic acid, Patinum and Radiation	Locally advanced head and neck SCC (oral cavity and oropharynx)	Intituto do Cancer do Estado de São Paulo	40	II	2012 2014	NCT01695122

SCC: squamous cell carcinoma HNSCC: head and neck squamous cell carcinoma

http://www.cancer.gov/clinicaltrials

Table 3

Histones and histones modifiers found mutated in HNSCC (NGS)

Gene Name	Gene Alias	UniProt	Protein Name	Protein Alias	cDNA Change	Mutation type	Protein Change
BCOR	KIAA1575	Q6W2J9	BCL-6 corepressor	BCoR	c.5486G>T	Nonsense	p.E1732
BCORL1		Q5H9F3	BCoR-L1	BCoR-like protein 1	c.3989G>A	Missense	p.R1292Q
CREBBP	CBP	Q92793	CREB-binding protein	---	c.4541G>A c.4765G>T c.1711C>T c.3053C>T c.964G>C	Missense Missense Nonsense Missense Missense	p.R1446H p.D1521Y p.Q503 p.T950M p.A254P
EHMT1	UHMTASE1, GLP, KIAA1876, KMT1D	Q9H9B1	EHMT1	Eu-HMTase1, GLP, GLP1, H3-K9-HMTase 5.	c.1157G>A c.168G>C	Missense Missense	p.D374N p.G44A
EHMT2	BAT8, C6orf30, G9A, KMT1C, NG36	Q96KQ7	EHMT2	Euchromatic histone-lysine N-methyltransferase 2, HLA-B-associated transcript 8, H3-K9-HMTase 3	c.1155C>T	Missense	p.S384L
EP300	P300	Q09472	p300 HAT	E1A-associated protein p300	c.4549G>T c.4590G>A c.4154G>T	Missense Missense Missense	p.C1385F p.D1399N p.C1385F
H2BFWT	---	Q7Z2G1	Histone H2B type W-T	H2B histone family member W testis-specific	c.451C>T	Missense	p.R143C
H3F3A	H3.3A, H3F3, H3.3B	P84243	H3.3	---	c.122C>T	Missense	p.R3C
H3F3C	---	Q6NXT2	H3.3C	H3.5	c.439A>G c.172A>G	Missense Missense	p.K122E p.T33A
HDAC3	---	O15379	HD3	RPD3-2, SMAP45	c.968G>T	Missense	p.R301L
HDAC4	KIAA0288	P56524	HD4	---	c.3452G>T	Missense	p.G887V
HDAC9	HDAC7, HDAC7B, HDRP, KIAA0744, MITR	Q9UKV0	HD9	HD7, HD7b, Histone deacetylase-related protein, MEF2-interacting transcription repressor MITR	c.2643C>A	Missense	p.H868N
HIST1H1B	H1F5	P16401	H1.5	H1a, H1b, H1s-3	c.727G>C	Missense	p.K225N
HIST1H1E	H1F4	P10412	H1.4	H1b, H1s-4	c.146G>A	Missense	p.G29D
HIST1H2AJ	H2AFE	Q99878	H2A type 1-J	H2A/e	c.89G>A c.56C>T	Missense Missense	p.R30Q p.S19F
HIST1H2BF	HIST1H2BC, HIST1H2BE, HIST1H2BG, HIST1H2BI	P62807	H2B type 1-C/E/F/G/I	H2B/a, H2B/g, H2B/h, H2B/k, H2B/l	c.229G>A c.152C>G	Missense Missense	p.E77K p.P51A
HIST1H2BH	H2BFJ	Q93079	H2B type 1-H	H2B/j	c.9T>G c.67C>A	Missense Missense	p.D3E p.Q23K

Gene Name	Gene Alias	UniProt	Protein Name	Protein Alias	cDNA Change	Mutation type	Protein Change
HIST1H3A	HIST1H3B, HIST1H3C, HIST1H3D, HIST1H3E, HIST1H3F, HIST1H3G, HIST1H3H, HIST1H3I, HIST1H3J	P68431	H3.1	H3/a, H3/b, H3/c, H3/d, H3/f, H3/h, H3/I, H3/j, H3/k, H3/l	c.125A>G c.90G>A c.120A>T c.103A>T	Missense Missense Missense Missense	p.Y42C p.R27Q p.K37M p.T33S
HIST1H4B	HIST1H4A, HIST1H4C, HIST1H4D, HIST1H4E, HIST1H4F, HIST1H4H, HIST1H4I, HIST1H4J, HIST1H4K, HIST1H4L, HIST2H4A, HIST2H4B, HIST4H4	P62805	H4	---	c.202C>T c.77A>T c.162G>C c.11G>A c.262G>T c.118C>G c.39A>T	Missense Missense Missense Missense Missense Missense	p.R68W p.N26I p.E54D p.R4K p.V88F p.R40G p.K13I
HIST2H2AB	---	Q8IUE6	H2A type 2-B	---	c.133G>A	Missense	p.G45R
HIST2H2BE	H2BFQ	Q16778	H2B type 2-E	H2B-GL105, H2B/q	c.247G>A	Missense	p.D69N
KDM3A	JHDM2A, JMJD1, JMJD1A, KIAA0742, TSGA	Q9Y4C1	Lysine-specific demethyla, se 3A	Jumonji domain-containing protein 1A	c.3698C>G c.4178C>A	Missense Nonsense	p.S1124C p.S1284
KDM4A	JHDM3A, JMJD2, JMJD2A, KIAA0677	O75164	Lysine-specific demethylase 4A	Jumonji domain-containing protein 2A	c.174C>T c.503G>A c.2150G>T	Missense Missense Missense	p.S3F p.E113K p.A662S
KDM4B	JHDM3B, JMJD2B, KIAA0876	O94953	Lysine-specific demethylase 4B	Jumonji domain-containing protein 2B	c.1418T>C	Missense	p.S398P
KDM5A	JARID1A, RBBP2, RBP2	P29375	Lysine-specific demethylase 5A	Jumonji/ARID domain-containing protein 1A, RBBP-2	c.5287G>C c.2708G>A	Missense Missense	p.G1642R p.R782Q
KDM5B	JARID1B, PLU1, RBBP2H1	Q9UGL1	Lysine-specific demethylase 5B	CT31, Histone demethylase JARID1B, Jumonji/ARID domain-containing protein 1B, PLU-1, RBP2-H1	c.1741A>T c.2368G>A	Missense Missense	p.Y542F p.C751Y
KDM5C	DXS1272E, JARID1C, SMCX, XE169	P41229	Lysine-specific demethylase 5C	Histone demethylase JARID1C, Jumonji/ARID domain-containing protein 1C, SmcX, protein Xe169	c.1539G>C	Missense	p.D336H
MYST1	KAT8, MOF	Q9H7Z6	MYST-1	Lysine acetyltransferase 8, MOZ, YBF2/SAS3, SAS2 and TIP60 protein1, hMOF	c.829C>G	Missense	p.L271V
MYST2	KAT7, HBO1, HBOa	O95251	MYST-2	KAT7, Histone acetyltransferase binding to ORC1 Lysine acetyltransferase 7, MOZ, YBF2/ SAS3, SAS2 and TIP60 protein 2	c.179C>T c.1016_1017delTT	Missense Frame_Shift_Del	p.S18F p.L297fs
MYST3	MOZ, KAT6A, RUNXBP2, ZNF220	Q92794	MYST-3	KAT6A, MOZ, YBF2/SAS3, SAS2 and TIP60 protein 3	c.3999G>T	Missense	p.W1152L
NAT10	ALP, KIAA1709	Q9H0A0	N-acetyltransferase 10		c.1598G>A	Missense	p.A452T
PHF21A	BHC80, KIAA1696	Q96BD5	PHD finger protein 21A	BHC80a BRAF35-HDAC complex protein BHC80	c.2553C>G c.2491C>T	Missense Missense	p.I643M p.R623C

Gene Name	Gene Alias	UniProt	Protein Name	Protein Alias	cDNA Change	Mutation type	Protein Change
RCOR1	KIAA0071, RCOR	Q9UKL0	REST corepressor 1	CoREST	c.e3_splice_site	Splice_Site_SNP	
RCOR2	---	Q8IZ40	REST corepressor 2	---	c.731A>T	Nonsense	p.K115
RCOR3	KIAA1343	Q9P2K3	REST corepressor 3	---	c.559A>T	Missense	p.M122L
TNP2	---	Q05952	TP-2	---	c.206C>A	Missense	p.S55R

Data represents HNSCC mutations by Stransky N. *et al*, and Agrawal *et al* supplemented by UniProt database (http://www.uniprot.org/), and COSMIC (http://cancer.sanger.ac.uk/cancergenome/project/cosmic/). Only missense and nonsense mutations along with frame shift deletions and splice site SNP of histones and histones associated modifiers were included. Synonymous mutations were excluded.