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Nitric Oxide-Mediated Histone Hyperacetylation in Oral Cancer: Target for a Water-Soluble HAT Inhibitor, CTK7A

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SUMMARY

Altered histone acetylation is associated with several diseases, including cancer. We report here that, unlike in most cancers, histones are found to be highly hyperacetylated in oral squamous cell carcinoma (OSCC; oral cancer) patient samples. Mechanistically, overexpression, as well as enhanced autoacetylation, of p300 induced by nucleophosmin (NPM1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) causes the hyperacetylation, which is nitric oxide (NO) signal dependent. Inhibition of the histone acetyltransferase (HAT) activity of p300 by a water-soluble, small molecule inhibitor, Hydrazinocurcumin (CTK7A), substantially reduced the xenografted oral tumor growth in mice. These results, therefore, not only establish an epigenetic target for oral cancer, but also implicate a HAT inhibitor (HATi) as a potential therapeutic molecule.

INTRODUCTION

Chromatin is a dynamic entity that plays a critical role in nucleus-related phenomena such as transcription, replication, and repair (Carrozza et al., 2003). Posttranslational modifications of chromatin play an important role in maintaining chromatin structure-function and hence regulate gene expression, cell growth, and differentiation. Increasing evidence suggests that chromatin structure-function has a pivotal role in several disease manifestations (Thorne et al., 2009). This is evident from the fact that genetic alterations and/or a more diverse group of epigenetic changes may result in cancer development and other diseases (Thorne et al., 2009; Fraga et al., 2005; Varambally et al., 2002; Seligson et al., 2005; Das et al., 2009; Pfister et al., 2008; Esteller, 2007; Bhaumik et al., 2007).

Of the various histone acetyltransferases (HATs), the global transcriptional coactivator p300 (Debes et al., 2003) has been observed to have altered expression in a few tumors. For example, some tumors show higher levels of p300 (Debes et al., 2003; Ishihama et al., 2007), some primary tumors and cell lines exhibit mutations in p300 acetyltransferase (Gayther et al., 2000), and loss of heterozygocity at the p300 locus has been shown to be associated with colorectal, breast, and brain cancer (glioblastoma) (Gayther et al., 2000; Van Beekum and Kalkhoven, 2007). Although these data indicate the involvement of the p300 gene, the enzyme (acetyltransferase) activity has not been established as the cause of malignancy (Van Beekum and Kalkhoven, 2007).

Recently, alterations of histone modifications have been reported in different cancers. The loss of Lys 16 acetylation and Lys 20 methylation of H4 were found to be associated with primary tumors and tumor cell lines (Fraga et al., 2005). In another study, changes in bulk histone modifications of cancer cells were found to be predictive of the clinical outcome of prostate cancer (Seligson et al., 2005). However, a rare exception of histone hyperacetylation has been observed in hepatocellular carcinoma (Bai et al., 2008). Apart from cancer, dysfunction of lysine acetyltransferases has been implicated in other diseases such as inflammatory processes, Huntington's disease, cardiac disease, and diabetes (Davidson et al., 2005; Zhou et al., 2004; Rouaux et al., 2003). These observations suggest that specific and relatively nontoxic inhibitors of acetyltransferases could be considered as new generation therapeutic agents for cancer. Although several HAT inhibitors have been discovered (Selvi and Kundu, 2009; Cole, 2008) with a potential therapeutic importance in HIV and cardiac disease (Davidson et al., 2005; Mantelingu et al., 2007; Balasubramanyam et al., 2004; Morimoto et al., 2008), the effect of HAT inhibitors on cancer manifestation has not yet been tested.

The hallmark of cancer is hyperproliferative cells, which have evaded the cellular apoptotic machinery and hence, exhibit overexpressed antiapoptotic proteins. NPM1 (also known as B23) (Grisendi et al., 2006; Shandilya et al., 2009) and GAPDH (Altenberg and Greulich, 2004) are two such genes that are known to be frequently upregulated in many cancers, including oral cancer. Both of these proteins are suggested to be positive regulators of cell proliferation.

Here, we report that histone H3 is hyperacetylated in oral cancer patient samples and is positively correlated with upregulated NPM1 and GAPDH protein levels. We also present a novel mechanism to explain how hyperacetylation of H3 could be regulated by NPM1 and GAPDH in a NO-dependent manner involving p300 acetyltransferase. Furthermore, we show that a novel water-soluble HATi, CTK7A, inhibits oral tumor cell growth in nude mice.

RESULTS

Histone H3 Is Hyperacetylated in Oral Cancer

To investigate the histone acetylation status in different cancers, histones were isolated from different cell lines and subjected to immunoblotting analysis with antiacetylated histone H3 (anti-H3AcK9AcK14) antibodies. We observed that histones are predominantly hyperacetylated in oral (KB, OSCC cell) and liver (HepG2) cancer cell lines (Figure 1A). Histone hypoacetylation is a hallmark of cancer, except hepatocarcinoma (Bai et al., 2008). The enhanced H3 acetylation in the oral cancer cell line was paradoxical yet interesting. These results, therefore, led us to examine the acetylation levels of histone H3 in the tissues obtained from grade II OSCC (tumor samples from this grade were readily available) patients by immunohistochemistry (IHC) (Figure 1B; also see Table S1 available online) and immunoblotting (Figure 1C) analyses using specific antibodies. The immunostaining revealed that histone H3 (predominantly H3K14) is hyperacetylated in the cancerous tissues in comparison to the adjacent normal tissue (Figure 1B). As H3K14 is the predominant in vivo target of p300-mediated acetylation (Kouzarides, 2007), we next investigated the expression level of p300, which was found to be significantly overexpressed in the malignant tumor as compared to the adjacent normal tissue (Figure 1B). Overexpression of p300 was also confirmed by real-time RT-PCR analysis of RNA isolated from patient samples (Figure S1). Other HATs such as CBP and PCAF showed no significant change as monitored by IHC and RT-PCR analysis (Figure 1B; Figure S1). These results suggest that highly active acetylated p300 could be involved in the histone hyperacetylation in malignant oral tumors. Because autoacetylation of p300 enhances its acetyltransferase activity (Thompson et al., 2004), the autoacetylation status of p300 was also verified using a polyclonal antibody, which specifically recognizes acetylated p300 (ac-p300) molecules (Thompson et al., 2004). Interestingly, p300 was found to be hyperacetylated in oral cancer samples (Figure 1B).

Autoacetylation of p300 could be enhanced by several factors (Thompson et al., 2004; Sen et al., 2008; Hansson et al., 2009; Turnell et al., 2005; Huang and Chen, 2005), some of which are overexpressed in different cancers. We found that GAPDH, an enhancer of p300 autoacetylation (Sen et al., 2008), is significantly overexpressed in the oral tumor tissues as compared with the control (Figure 1B). As reported recently (Shandilya et al., 2009), a concomitant increase of NPM1 was also observed in these patient samples (Figure 1B). Subsequently, we took six different pairs of tissue samples (tumor and corresponding adjacent normal tissue) and determined the levels of protein overexpression by immunoblotting analyses. In all of the samples, histone H3 was hyperacetylated as probed by anti-H3AcK9AcK14 acetylation-specific antibody (Figures 1B and 1C). Interestingly, we also observed the hyperacetylation of H2AK5 and H3K56 (Figures 1B and 1C), other p300-specific sites which suggest further the role of p300-mediated acetylation. The levels of acH4K16 (Figures 1B and 1C) were found to be significantly low in the oral tumor samples, as observed in several cancers (Fraga et al., 2005). However, the H4K8 acetylation was minimally altered (Figures 1B and 1C). Furthermore, GAPDH and NPM1 were also found to be overexpressed in all of the tumor tissue samples (Figures 1B and 1C). Collectively, these data suggest that the overexpression of GAPDH and NPM1 are positively correlated to histone hyperacetylation in oral cancer. An interesting question raised at this juncture: is there any systematic molecular correlation of p300 autoacetylation and overexpression of these proteins that could be cell signal driven?

NO-Induced Histone Acetylation is Associated with NPM1 and GAPDH Overexpression via p300 Autoacetylation

The free radical gas NO is generated by the nitric oxide synthase (NOS) family of enzymes. NO is a pleiotropic signaling molecule that has been identified as a mediator for numerous physiological and pathophysiological conditions (Moncada et al., 1991). Because increased production of NO was noticed in oral cancer with a simultaneous upregulation of inflammatory (predominantly, NFk-B-responsive) genes (Gallo et al., 1998; Czesnikiewicz-Guzik et al., 2008), we hypothesized that NO signaling could be the link to overexpression of NPM1 and GAPDH, which in turn induces autoacetylation of p300, followed by hyperacetylation of histones. Immunohistochemical analysis revealed that indeed the inducible nitric oxide synthase (iNOS) levels were significantly enhanced in tumor tissue samples (Figure 2A). COX2 levels were also found to be higher in these tumor tissue samples (Figure 2A). A recent report implicates NO-dependent, nuclear localized GAPDH as an enhancer of p300 autoacetylation and thereby its catalytic activity (Sen et al., 2008). In the oral cancer patient samples analyzed, GAPDH was predominantly localized to the nucleus (Figure 2A). To investigate the causal relationship of iNOS and overexpression of NPM1 and GAPDH, KB cells were treated with the NO donor, S-nitrosoglutathione (GSNO). It was observed that the expression of both NPM1 and GAPDH was enhanced by GSNO in a concentration-dependent manner (Figure 2B; see also Figure S2). In agreement with the previous report (Sen et al., 2008), we also found that GAPDH is acetylated in a NO-dependent manner (Figure 2C). Interestingly, acetylation of NPM1 was also dramatically enhanced by GSNO treatment in KB cells (Figure 2C). Similar results were observed upon GSNO treatment in HeLa and KOSC-2 cells (see Figure S2).

In order to gain insight into signaling pathways, we investigated the effect of interferon-γ (IFN-γ) on NPM1 and GAPDH acetylation in KB cells, as it is known to activate iNOS gene expression to produce NO (Fukumura et al., 2006). We found that IFN-γ efficiently enhanced the NPM1 and GAPDH acetylation in a NO-dependent manner in KB (Figure 2D), HeLa, and KOSC-2 cells (Figure S2), which was abolished or reduced in the presence of a specific iNOS inhibitor, N-(3-(Aminomethyl) benzyl)acetamidine (1400 W). Furthermore, we found that IFN-γ treatment could induce the translocation of the cytosolic protein GAPDH to the nucleus of KB cells (Figure 2E) and HeLa and KOSC-2 cells (Figure S2). Taken together, these results suggest the involvement of NO signaling in the overexpression of NPM1 and GAPDH and their acetylation in KB cells.

GAPDH, which is positively regulated by NO signaling, enhances the autoacetylation of p300 (Sen et al., 2008) and presumably leads to histone hyperacetylation. These observations prompted us to investigate the role of NPM1 in the activation of p300 (autoacetylation). p300 autoacetylation reaction was performed in the presence of NPM1 and ^{3}H-acetyl-CoA. NPM1