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Monoamine Oxidase A is Highly Expressed in Classical Hodgkin Lymphoma

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Abstract

Monoamine oxidase A (MAOA) is a mitochondrial enzyme that catalyzes oxidative deamination of neurotransmitters and dietary amines and produces H₂O₂. It facilitates the progression of gliomas and prostate cancer, but its expression and functional relevance have not been studied in lymphoma. Here, we evaluated MAOA in 427 cases of Hodgkin and non-Hodgkin lymphoma and in a spectrum of reactive lymphoid tissues by immunohistochemistry on formalin-fixed paraffin-embedded specimens. MAOA was expressed by Hodgkin Reed-Sternberg (HRS) cells in the majority of classical Hodgkin lymphomas (cHL) (181/241; 75%), with 34.8% showing strong

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Author Contributions Statement

Conceived study: JCS. Contributed to study design: JCS, PCL, INS, WC, AM, CS, CHW. Performed the experiments: PCL. Analyzed the data: PCL, INS, CHW and JCS.

Clinical samples Analyzed: PCL, INS, EL and AM. Contributed clinical samples: INS, EL, CS and WC. Wrote, reviewed and edited the paper: JCS, PCL, INS, AM, CS and WC.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods **NO**

expression. Weak MAOA was also noted in a minority of primary mediastinal large B-cell lymphomas (8/47; 17%) and in a mediastinal gray zone lymphoma. In contrast, no MAOA was found in non-neoplastic lymphoid tissues, nodular lymphocyte predominant Hodgkin lymphoma (0/8) or any other non-Hodgkin lymphomas studied (0/123). MAOA was more common in Epstein-Barr virus (EBV)-negative compared to EBV-positive cHL ($P < 0.0001$) and was especially prevalent in the EBV-negative nodular sclerosing subtype. Similar to primary human lymphoma specimens, most cHL-derived cell lines displayed MAOA activity, whereas non-Hodgkin-lymphoma derived cell lines did not. The MAOA inhibitor clorgyline reduced the growth of L1236 cells and U-HO1 cells, and shRNA knockdown of MAOA reduced the growth of L1236 cells. Conversely, ectopic overexpression of MAOA increased the growth of MAOA-negative HDLM2 cells. Combined treatment with clorgyline and ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) was more effective in reducing cell growth than either regimen alone. In summary, MAOA is highly expressed in cHL and may reflect the distinct biology of this lymphoma. Further studies on the potential utility of MAOA as a diagnostic marker and therapeutic target are warranted.

Keywords

monoamine oxidase A (MAOA); classical Hodgkin Lymphoma; Hodgkin Reed-Sternberg (HRS) cells

Introduction

Monoamine oxidase A (MAOA) is a mitochondrial enzyme that catalyzes the oxidative deamination of monoamine neurotransmitters (serotonin, norepinephrine and dopamine) and dietary amines (tyramine) [1, 2]. MAOA has been extensively studied in the context of neuropsychiatric disorders including anxiety [3], aggressive behavior [4] and autism spectrum disorders [5–8]. Recently, overexpression of MAOA has been reported in prostate cancer [9, 10], renal cell carcinoma [11] and glioma [12]. A significant correlation exists between increased MAOA expression and high Gleason grade in prostate cancer [13, 14]. Furthermore, it has been suggested that MAOA facilitates cancer progression by promoting epithelial to mesenchymal transition and activation of the AKT/FOXO1 signaling pathway [10].

Classical Hodgkin lymphoma (cHL) is one of the most common types of lymphoma occurring among adolescents and young adults in industrialized countries, with a peak incidence age of 22 years [15, 16]. There are approximately 9,100 cases diagnosed in the U.S.A. each year (www.seer.cancer.gov). Although the 5-year overall survival (OS) rate is high, approximately 85%, treatment-related adverse effects include a high lifetime risk of secondary cancers, cardiovascular disease and pulmonary fibrosis, resulting in increased mortality over time [17]. Standard multimodality treatments, which include ABVD [18, 19] (doxorubicin, bleomycin, vinblastine, dacarbazine) chemotherapy and IFRT (involved field radiotherapy) [20], leads to cures in most patients, but about 20% remain refractory to treatment or relapse following the initial treatment response.

cHL is unique among cancers in that the neoplastic cells - mononuclear Hodgkin cells and bi- or multi-nucleated Reed-Sternberg cells (HRS cells) - typically comprise less than 1% of the tumor, whereas the tumor bulk is composed of non-neoplastic inflammatory cells [21, 22]. Based on distinct tumor microenvironment patterns and neoplastic cell appearance, cHL is further subdivided into 4 histologic subtypes: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte depleted (LD) and lymphocyte-rich (LR) [23, 24]. Epstein-Barr virus (EBV) DNA can be seen in the neoplastic HRS cells in a subset of cHL cases and is more frequently observed in the MC subtype compared to NS [25–27]. However, this association is imperfect and there is a lack of specific markers for biologic differentiation of the histologic and epidemiologic subtypes of cHL.

MAOA expression has not been studied in cHL or other lymphoma subtypes. Here, we examined MAOA in primary tissue specimens of cHL, various non-Hodgkin lymphoma (NHL) subtypes, and reactive lymph node samples. We also characterized MAOA expression and the effects of MAOA inhibition in lymphoma-derived cell lines.

Material and Methods

Primary patient specimens

Formalin-fixed paraffin embedded (FFPE) tissues from 427 cases were evaluated. Diagnostic pretreatment specimens of cHL patients (241 cases total) were represented on tissue microarrays (TMA) that included 91 females and 150 males, with a median age of 33.5 years (range 13 – 82 years). Of these, 88 cHL patients were diagnosed at the Los Angeles County-University of Southern California Medical Center (LAC+USC), Los Angeles, CA and 153 cHL patients were diagnosed at the British Columbia Cancer Agency (BCCA) [28]. Among the 88 cases from LAC+USC, 65% were Hispanic and foreign-born, therefore the usual equal male:female ratio of young adult cHL was not seen. Among Hispanics born in Mexico/Central America, there was a higher male (n=64): female (n=24) ratio, the age at diagnosis was slightly older, and EBV in HRS cells was more prevalent, which enabled us to examine differences in EBV-positive and EBV-negative tumors in sufficient numbers.

In addition to cHL, MAOA immunohistochemistry was also performed on nodular lymphocyte predominant Hodgkin lymphoma (n=8) from Keck Medical Center of the University of Southern California and 169 non-Hodgkin lymphoma (NHL) cases including primary mediastinal large B-cell lymphoma (PMBL, n=47) from BCCA, diffuse large B-cell lymphoma (DLBCL, n=54), follicular lymphoma (FL; n=33), Burkitt lymphoma (BL; MCL; n=19), mantle cell lymphoma (n=13), mediastinal gray zone lymphoma (intermediate between cHL/DLBCL, n=1), EBV+DLBCL (n=1), and T-cell/histiocyte-rich large B cell lymphoma (THRLBCL, n=1) diagnosed at the USC Keck Medical Center and Los Angeles County Medical Center.

A variety of non-neoplastic lymphoid tissues were examined, including tonsil (n=3) and lymph nodes (n=4) with varying degrees of immunoblastic hyperplasia. Reed-Sternberg (RS)-like cells seen in settings other than cHL were evaluated, including small lymphocytic

lymphoma (SLL) with RS-like cells (n=1) and angioimmunoblastic T-cell lymphoma (AITL) with RS-like cells (n=1).

This study was approved by the Institutional Review Board (IRB) of the University of Southern California (HS10-260) and the University of British Columbia–BCCA. Specimens were reviewed for adequacy and diagnoses were confirmed using the World Health Organization classification criteria [21].

Immunohistochemistry and EBV in situ hybridization

Immunohistochemical staining was performed on the Leica Bond III staining platform using heat induced antigen retrieval and an universal polymer detection system with 3, 3'-diaminobenzidine (DAB) as the chromogen. MAOA (#H-70, rabbit polyclonal, Santa Cruz, Dallas, TX, USA) and CD30 (#JCM182, Leica Biosystems, Newcastle, UK) immunohistochemistry was performed as described previously [10]. All cases were evaluated and scored by two board-certified hematopathologists (INS and AM). MAOA staining intensity was assessed semi-quantitatively as 0 (no expression) to 3+ (strong expression) and the percentage of positive tumor cells was recorded in 10% increments. *In situ* hybridization (ISH) for EBV encoded RNA (EBER) was performed using the Novocastra™ Epstein-Barr virus ISH Kit [Ready-to-use (RTU), Leica Microsystems, Inc. Buffalo Grove, IL, USA], which uses a pre-diluted fluorescein-conjugated oligonucleotide supplied in hybridization solution for FFPE tissue sections.

Cell lines and reagents

Human lymphoma cell lines include cHL-derived (L1236, U-HO1, SUP-HD1, L591, L428, HDLM2, L540, and KM-H2), NLPHL-derived (DEV) and NHL/acute leukemia-derived cell lines (SU-DHL-6, SU-DHL-10, Toledo, U937, JeKo-1, NU-BL-1, DAUDI, Jurkat, and a pre-B acute lymphoblastic leukemia). All cells were cultured in RPMI-1640 (Corning cellgro®, MA, USA) containing 10% to 20% fetal bovine serum and 100 µg/mL penicillin/streptomycin in 5% CO₂ incubator at 37 °C, with the exception of U-HO1 cells that were cultured in a 4:1 mixture of 80% Iscove's Modified Dulbecco's Media (Thermo Fisher Scientific Inc., Wilmington, MA, USA) and RPMI-1640 containing 20% FBS plus 2mM L-glutamine. SUP-HD1 cells were cultured in 80% McCoy's 5A (Thermo Fisher) with 20% FBS. ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) were purchased from Sigma Aldrich (St. Louis, MO, USA).

MAOA catalytic activity assay

MAOA catalytic activity was determined as described previously [10]. In brief, cell homogenates were incubated with 1 mM [¹⁴C] 5-HT at 37 °C for 20 min. The reaction product was extracted and radioactivity determined by a scintillation counter (LS 6500, Beckman Coulter, Inc., Brea, CA, USA).

Cell viability, cell growth and colony formation assays

Cell viability was determined by MTS assays per the manufacturer's instruction (Promega, Madison, WI, USA). 5×10³ cells were seeded in triplicate and incubated with drugs at various concentrations for the indicated time periods. MTS reagent (20 µl/well) was added to

each well and incubated for 4 h at 37 °C, 5% CO₂ and the results were analyzed by absorbance at 490 nm with a microplate reader Synergy HTX (Bio-Tek, Winooski, VT, USA). To measure cell growth, 2×10⁵ L1236 or U-HO1 cells were seeded in each well and incubated with clorgyline for various time periods. Cells were then mixed with 0.4 % Trypan Blue Stain (Thermo Fisher) and cell numbers counted using a hemocytometer. For colony forming assays, 5×10³ cells (L1236 or U-HO1 cells) were seeded and treated with clorgyline at various concentrations for 48 h. The culture medium included 10% FBS and 0.8% methylcellulose. The medium was removed and replaced with a fresh medium every other day for 21 days. Colonies were visualized by staining with 1% methylene blue and counted.

shRNA mediated knock-down of MAOA in L1236 cells

The human *MAOA* gene was silenced in L1236 cells through RNA interference by electroporating shRNA using the Gene Pulser Xcell System (Bio-Rad Laboratories, Hercules, CA, USA) using 140 volts and capacitance of 1000 µF per manufacturer's instruction. The shRNA targeting *MAOA* (CGGAUAUUCUCUGUCACCAAUCUCGAGAUUGGUGACAGAGAAUAUCCGUU) was purchased from Sigma-Aldrich (#NM_000240_TRCN0000046009 [10]. A scrambled version of the above shRNA sequence was used as a negative control.

Overexpression of MAOA

To express the human *MAOA* gene in MAOA-negative HDLM2 cells, we employed electroporation as described above. A human MAOA expressing plasmid was constructed by inserting the human *MAOA* gene coding sequence at the EcoRI-Bgl II cloning sites in 3xFLAG-pCMV vector (Sigma-Aldrich) containing a neomycin-resistance gene. Successful MAOA knockdown or overexpression was verified by western blot analysis and MAOA catalytic activity assay.

Western blot analysis

Western blot analysis was performed as described previously [29]. Proteins in whole-cell lysates were resolved by SDS-PAGE and transferred onto PVDF membranes using the Mini Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with anti-human MAOA or GAPDH (Santa Cruz) antibodies, and with secondary antibody conjugated to horseradish peroxidase (IgG-HRP). The signals were visualized using the Pierce ECL Western Blotting substrate (Thermo Fisher) and imaged with ChemiDoc Touch imaging system (Bio-Rad). The band intensity was measured using Image Lab (Bio-Rad). GAPDH was used as loading control to normalize the signals of MAOA.

Statistical Analysis

All data are presented as mean ± standard error of mean (SEM) values and analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). One-way ANOVA, Mann-Whitney U-test and chi-square test were performed to compare groups. When the number in the cell was < 5, a Fisher's exact test was used. A *p*-value of < 0.05 was considered statistically significant.

Results

MAOA is highly expressed by HRS cells in the majority of classical Hodgkin lymphomas

We examined MAOA expression in archived FFPE tissues from cHL patients. A representative NS cHL case is shown in Figure 1A. MAOA was highly expressed by neoplastic HRS cells as indicated by distinct membrane and cytoplasmic immunoreactivity (Figure 1B). Additionally, a focal perinuclear staining pattern was also occasionally observed (Figure 1C). Dual MAOA/CD30 immunostaining confirmed the presence of MAOA in HRS cells (Figure 1D). Among the 241 cHL cases analyzed, 75% (181 of 241) expressed MAOA in HRS cells, with 34.8% (84 of 241) showing strong, uniform (3+) MAOA expression, while moderate (2+) or weak (1+) staining in HRS cells was seen in 19.5% (47/241) and 20.7% (50/241), respectively (Figure 2A). While a spectrum of immunoreactivity was apparent, most cases were polarized towards either strong expression or negativity for MAOA. By cHL histologic subtype, MAOA expression was most prevalent in NS cases (139/181), followed by MC (23/38), LD (4/7), LR (3/6), and NOS (5/9) (Figure 2B).

In cHL cases, the non-neoplastic cells in the background infiltrate were mostly negative for MAOA expression, although weak (1+) to moderate (2+) levels of immunoreactivity were observed in a minority of cases, mostly in macrophages and rarely in benign lymphocytes. Weak (1+) endothelial cell staining in blood vessels was observed in all cases and served as an internal positive control.

No MAOA staining was seen in the lymphoid compartments of reactive lymphoid tissues, including germinal center B-cells and interfollicular immunoblasts, including cases with prominent immunoblastic hyperplasia (e.g. viral lymphadenitis) (Figure 1E, F; Figure 2B). As seen in the lymphoma cases, weak endothelial cell staining served as an internal positive control (Figure 1E, inset).

To determine the specificity of MAOA expression in cHL, we also examined a spectrum of other lymphoma subtypes. No MAOA staining was seen in neoplastic or non-neoplastic cells of 8 NLPHL tumors or in 119 cases of non-Hodgkin lymphoma, including DLBCL, FL, BL or MCL (Figure 1G, H; Figure 2B). Interestingly, while most cases of PMBL were negative for MAOA, a minority (8 cases, 17%) showed weak and variable expression in the tumor cells (Figure 1I; Figure 2B). In most of these cases, the percentage of positive tumor cells was below 50%. Furthermore, a single case of mediastinal gray zone lymphoma (with features intermediate between Hodgkin lymphoma and DLBCL) also showed partial/weak MAOA expression in the malignant cells (Figure 1J). This contrasts with the higher proportion of cHL cases that were MAOA-positive (75%).

Correlation of MAOA expression with EBV status

The EBV status of 241 cHL cases was assessed by EBER *in situ* hybridization. The EBV status was distributed as follows: 32/181 (17.7%) NS cases, 11/38 (28.9%) MC cases, 1/6 (17%) LR cases, 1/7 LD cases (14.3%) and 3/9 (33.3%) of NOS cases were EBV-positive. As previously reported, MC had the highest proportion of EBV-positive cases [26, 27]. The percentage of HRS cells expressing MAOA protein by IHC was enumerated on a

scale from 0% (no MAOA+ HRS cells) to 100% (all HRS cells MAOA+), in 10% increments. Overall, more EBV-negative cases (147/193, 76%) demonstrated MAOA protein in (any) HRS cells compared to EBV-positive cases (27/48, 56%) ($p = 0.001$). The difference was more pronounced when comparing those cases with the highest percentage of MAOA+ HRS cells; 79/193 (41%) of EBV-negative compared to 6/48 (13%) of EBV-positive cases had 80% MAOA+ HRS cells ($p = 0.0004$). Using a non-parametric comparison method, the median percentage of HRS cells expressing MAOA protein was 50% in EBV-positive and 10% in EBV-negative-cases ($P=0.001$) (Figure 3).

When examined by both EBV status and histologic subtype (NS and MC), there were clear differences (Table 1). Among NS cases, (any) MAOA staining was more common among EBV-negative compared to EBV-positive cases (80% versus 62.5%, respectively, $p=0.0048$). However, there was no significant difference in MAOA by EBV tumor status among MC cases ($p=0.48$). Tumors with the highest proportion of MAOA+ HRS cells (80%) were more likely to be EBV-negative NS (48%) compared to EBV-positive NS (12.5%) ($p=0.0001$). Again, there was no difference in the proportion of cases with 80% MAOA+ HRS cells among EBV-negative (18%) and EBV-positive (18.1%) MC. Examined another way, the median percentage of HRS cells expressing MAOA by EBV and histological subtype was 70% for EBV-negative NS, 20% for EBV-positive NS, 20% for EBV-negative MC and 10% for EBV-positive MC. There was only one EBV-positive case in each of the LR and LD histological subtypes; neither had detectable MAOA in HRS cells.

Thus, we find that MAOA expression is more common among EBV-negative compared to EBV-positive cHL, and that the highest levels of MAOA are concentrated in the NS-EBV-negative subgroup.

MAOA catalytic activity is present in a subset of cHL-derived cell lines but not in other lymphoma cell lines

Prompted by our protein expression data obtained from primary lymphoma specimens, we examined MAOA activity in various cell lines. Most cHL-derived cell lines (L1236, SUP-HD1, U-HO1, L591 and L428) displayed MAOA activity, whereas NHL-derived cell lines did not. Other lymphoma cell lines that did not show MAOA activity include the NLPHL-derived cell line DEV, pre-B acute lymphoblastic leukemia, T cell leukemia (Jurkat), lymphoblastoid (NU-BL-1), and NHL cell lines (SU-DHL-6, SU-DHL-10, Toledo, U937, JeKo-1 and DAUDI) (Figure 4).

The MAOA inhibitor clorgyline reduces the growth of L1236 and U-HO1 cells

The effects of the MAOA specific inhibitor clorgyline on cell viability, cell number and colony formation were determined in MAOA-expressing (L1236 and U-HO1) and MAOA non-expressing (HDLM2) cell lines. Figure 5A shows that clorgyline reduced the viability of L1236 cells with an IC_{50} of 10 μ M. Growth was similarly inhibited in U-HO1 cells (Figure 5B). Quantitative data on colony numbers showed that clorgyline suppresses colony formation in a dose-dependent manner (Figure 5C, 5D). In contrast, clorgyline did not inhibit the cell growth of MAOA-negative HDLM2 cells (Figure S1). Taken together, these data indicate that clorgyline selectively reduces the growth of MAOA expressing cell lines.

To test the hypothesis that suppressing MAOA expression decreases cell growth, L1236 cells were generated that constitutively express shRNA directed against *MAOA* mRNA (shMAOA). Successful knockdown (KD) of MAOA was confirmed by Western blot analysis and MAOA catalytic activity assay (Figure 5E). MAOA protein levels were significantly reduced and catalytic activity was inhibited by ~60% ($p<0.001$) in shMAOA expressing cells compared to control vector-expressing cells (shCon) (Figure 5E). Cell growth, as determined by cell number assays, was also significantly reduced in shMAOA expressing cells compared to control cells ($p<0.05$; Figure 5E). Conversely, to determine if MAOA overexpression could increase cell growth, we generated HDLM2 cells expressing MAOA. Successful overexpression was confirmed by Western blot analysis and MAOA catalytic activity assay (Figure 5F, $p<0.001$). Cell growth, as determined by cell number, was also significantly increased in MAOA overexpressing cells compared to the control vector cells (vector, $p<0.01$). (Figure 5F).

Next, we investigated the effect of clorgyline in combination with standard ABVD treatment in cHL-derived cells. Untreated (control) cells were defined as 100%; all values were normalized to the control group. L1236 cells showed 50% inhibition of cell growth for the clorgyline and ABVD combination group ($p<0.01$), 28% inhibition for the ABVD alone group ($p<0.01$) and 25% inhibition for the clorgyline alone group ($p<0.01$; Figure 5G). U-HO1 cells showed similar results (Figure 5H). Our results suggest that combined treatment of ABVD with clorgyline significantly reduces cell growth *in vitro*.

Discussion

Increased MAOA expression has been reported in several types of solid tumors with potential biologic, prognostic and therapeutic consequences [9–12]. This is the first study to demonstrate MAOA protein expression by neoplastic HRS cells in a significant proportion of cHL and not in reactive lymphoid tissues, NLPHL, or a variety of NHL subtypes (except for a minor subset of PMBL). Specifically, MAOA is highly expressed by neoplastic HRS cells in 75% of cHL cases (34.8% with strong/uniform expression), most prevalent in the NS subtype. Furthermore, MAOA expression is more common in EBV-negative cHL cases regardless of histologic subtype.

cHL continues to be classified separately from NHL due to its unique clinical presentation, epidemiological risk pattern and histopathologic features. NS cHL is by far the most common cHL subtype in industrialized countries, accounting for 60–70% of cases, where it peaks in occurrence in young adults, is typically EBV-negative and is associated with higher socioeconomic status [16, 30]. There is evidence that cHL subtypes differ etiologically and possibly clinically (outcome) by histology, age at onset and/or EBV status, but there is overlap between these categories, thus the current criteria are not adequate for precise classification [16, 24, 31, 32]. In the present study, the higher prevalence of MAOA expression in EBV-negative NS suggests the possibility of selective activation of MAOA-dependent pathways in this subset of cHLs, which could provide additional criteria for subtype classification.

Whilst most other B-cell lymphomas are uniformly negative for MAOA in neoplastic cells, a minority of PMBLs (17%) showed weak/partial immunoreactivity. Moreover, a single case of mediastinal gray zone lymphoma (with features intermediate between cHL and DLBCL) also showed weak/variable expression. This is an intriguing result given the known biological relationships between PMBL and cHL [33, 34], the NS subtype in particular, and further underscores the potential role of MAOA in these related lymphomas (NS, EBV-negative cHL, and PMBL-like lymphomas).

Of interest, MAOA expression was not observed in mimics of cHL with RS-like cells, including small lymphocytic lymphoma and angioimmunoblastic T-cell lymphoma with RS-like cells. Whilst MAOA is not a highly sensitive marker for HRS cells, given that approximately a quarter of cHL cases are negative, further study is necessary to determine if there is a distinct biology among MAOA-positive cases compared to negative cases.

Similar to the findings in human specimens, MAOA catalytic activity was found in most cHL-derived cell lines (L1236, U-HO1, SUP-HD1, L591 and L428) but not in NHL or leukemia cell lines. Moreover, clorgyline, an MAOA inhibitor, reduced the growth of MAOA-positive L1236 and U-HO1 cells, but had no effects on the viability of MAOA-negative HDLM2 cells. We also found that L1236 cell growth was reduced by MAOA shRNA knockdown in L1236 cells. On the other hand, overexpression of MAOA in MAOA-negative HDLM2 cells increased their growth.

Notably, clorgyline showed greater efficacy in lymphoma cells (IC_{50} : 10 μ M for L1236 and 17.3 μ M for U-HO1) than prostate cancer cells (IC_{50} : 80.7 μ M for LNCaP, 113.5 μ M for C42B) [9]. Recently, we have shown that combined use of clorgyline with low dose temozolomide (TMZ) (current standard treatment) reduced brain tumor progression and increased survival in a murine xenograft model of glioma [12]. It also reduced the toxicity of TMZ. Others showed that combining MAOA inhibitors with a survivin inhibitor was more efficacious in treating prostate cancer [35]. ABVD chemotherapy is the first-line standard treatment for HL [18, 19, 36]. In this study, we showed that combining clorgyline with ABVD reduces cell growth more effectively than clorgyline or ABVD alone, suggesting that clorgyline may enhance the therapeutic efficacy of ABVD. The results suggest that MAOA may play a tumorigenic role in a significant subset of cHL and that MAOA inhibitors may have a therapeutic role in this setting. Intriguingly, procarbazine [37] (an alkylating agent which has a weak MAOA inhibitory effect) was historically used in combination with mechlorethamine, vincristine, and prednisone (MOPP) [38, 39] for cHL therapy with variable success before being replaced by ABVD, though the underlying mechanism of the procarbazine effect in this disease was not well understood.

We did not find a statistically significant difference in clinical outcome (overall survival) in relation to MAOA expression (data not shown). However, clinical outcome information was only available for a subset of the cHL cases (153 out of 241 cases) and those had been previously selected and enriched for treatment failure [28]. Further studies are necessary to determine the prognostic impact of MAOA activity in cHL in patients treated with standard regimens as well as with therapies that target MAOA.

In summary, we showed that MAOA is highly expressed by HRS cells in the majority of cHL, a minority of PMBL, and not in other non-Hodgkin lymphomas. In cHL, MAOA expression correlates with NS and EBV-negative subtypes. The mechanisms of MAOA upregulation in cHL, the biological consequences of its activation in HRS cells, the effects on the cHL tumor microenvironment, and the potential therapeutic role of MAOA inhibitors are under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Monoamine oxidase A (MAOA) is highly expressed by Hodgkin/Reed-Sternberg (HRS) cells of classical Hodgkin lymphoma (cHL)
 MAOA was detected by immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) patient material. (A) A representative nodular sclerosis cHL case, showing nodular aggregates of mixed inflammatory cells and scattered HRS cells (H&E stain, 100 $\times$. Inset 400 $\times$.) (B) MAOA IHC highlights HRS cells with distinct membrane and cytoplasmic staining (H&E stain, 100 $\times$. Inset 400 $\times$). Arrow indicates a MAOA-positive HRS cell (inset). (C) Alternative patterns of MAOA immunoreactivity in cHL: a case with focal perinuclear staining of HRS cells (arrow) is shown. (400 $\times$) (D) Double staining for CD30 (red) and

MAOA (brown) confirms localization of MAOA in HRS cells.(1000×) (E, F) MAOA IHC on reactive lymphoid tissues, including tonsil and normal lymph node, respectively (H&E stain, 100×, Inset 400×). No MAOA staining is observed in reactive lymphoid compartments. Weak (1+) staining of vascular endothelial cells is present (inset) as well as partial/weak staining of tonsillar crypt epithelium. (G, H) MAOA in non-Hodgkin lymphoma. No staining is observed in a variety of non-Hodgkin's lymphomas, including FL (G) and NLPHL (H) [H&E stain, 400×]. Weak endothelial cell staining (G) serves as an internal positive control. (I, J) [H&E stain, 400×] Among lymphomas other than cHL, only a minor subset of PMBL (I) and a mediastinal grey zone lymphoma (cHL/DLBCL) (J) show weak/partial staining of tumor cells (arrow).

Figure 2. MAOA is highly expressed in cHL and a minor subset of PMBL but not in NHLs or reactive lymphoid tissue

(A) Distribution of MAOA expression and staining intensity by IHC in 241 clinical samples.

*Each circle represents one case. Total of 241 cases. (B) Comparison of MAOA expression among cHL histologic subtypes, NHL cases, reactive lymphoid tissues, and lymphomas with RS-like cells. Abbreviations: primary mediastinal large B-cell lymphoma (PMBL); Epstein-Barr virus positive diffuse large B-cell lymphoma (EBV+DLBCL); T-cell/histiocyte-rich large B cell lymphoma (THRLBCL); small lymphocytic lymphoma with Reed-Sternberg-like cells (SLL with RS-like cells); angioimmunoblastic T-cell lymphoma (AITL with RS-

like cells). * A single case of mediastinal gray-zone lymphoma was also studied and is not shown (see text).

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Figure 3. MAOA expression in HRS cells is more frequent in EBV-negative cHL

We examined a total of 241 cases of cHL with EBV status: EBV-negative, n=193, EBV-positive, n=48. The *p*-value was calculated by Mann-Whitney U-test ($p < 0.001$) and Chi-square test ($p = 0.001$ EBV-negative compared to EBV-positive).

Figure 4. MAOA catalytic activity was present in classical Hodgkin lymphoma-derived cell lines but not in non-Hodgkin lymphoma-derived or leukemia-derived cell lines. The MAOA catalytic activity in cell lines derived from cHL, NHL and leukemia was determined.

Figure 5. The effect of MAOA inhibitor clorgyline on classical Hodgkin lymphoma derived L1236 or U-HO1 cell growth

(A) Clorgyline reduced the viability of L1236 or U-HO1 (B) cells. Cells were seeded and incubated with clorgyline (0–100 μ M) for 96 h, and then MTS assay was performed. (C, D) Clorgyline reduced the colony formation by L1236 (C) or U-HO1 (D) cells. Cells were seeded and treated with clorgyline (0, 1, 10 μ M), and then fed every two days for 21 days. Representative data are shown (magnification: 200 $\times$). (E) Growth was decreased (* p <0.05) in MAOA-targeting shRNA (shMAOA) compared to control (shCon) transfected L1236 cells. MAOA catalytic activity was reduced (** p <0.001) in shMAOA compared to

shCon. (F) Growth was increased (** $p<0.01$) in MAOA overexpressing HDLM2 cells compared to control vector-transfected cells. MAOA catalytic activity was increased (** $p<0.001$) in MAOA compared to vector control. MAOA reduction in (E) or overexpression in (F) was confirmed by Western blot and MAOA catalytic activity assay. GAPDH was used as loading control to normalize the signals of MAOA. All experiments were repeated three times. (G) Combined treatment of clorgyline and ABVD. Cells (L1236 or U-HO1) were seeded as in (A) and pre-incubated with clorgyline (1 μ M) for 48 h. Next, ABVD was added for 72 h and then MTS assay was performed. The average proliferation values of untreated control samples were taken as 100%. Data represent the mean $\pm$ SEM; n=3 experiments. (* $p<0.05$, ** $p<0.01$, clorgyline+ ABVD compared to control; # $p<0.05$, ## $p<0.01$, clorgyline+ ABVD compared to ABVD alone; <0.05, && $p<0.01$, clorgyline+ ABVD compared to clorgyline alone group.)

Table 1

Percent of Hodgkin Reed-Sternberg cells expressing MAOA by Epstein-Barr virus status and histology in nodular sclerosis and mixed cellularity classical Hodgkin lymphoma tumors.

	NS (n=181)		MC (n=38)		P-value	P-value
	EBV+ (n=32)	EBV- (n=149)	EBV+ (n=11)	EBV- (n=27)		
MAOA (% of Cells Stained)*						
0	12 (37.5%)	29 (20%)	5 (45.5%)	8 (30%)		
>0% (Any)	20 (62%)	120 (80%)	6 (54.5%)	19 (70%)	0.048 ^a	0.46 ^a
1-39	8 (25%)	27 (18%)	4 (36.4%)	10 (37%)		
40-79	8 (25%)	21 (14%)	0 (0%)	4 (15%)		
80-100	4 (12.5%)	72 (48%)	2 (18.1%)	5 (18%)	0.001 ^b	0.64 ^b

^aP value comparing 0 versus >0%

^bP value comparing 0, 1-39, 40-79, 80-100

Values in bold show statistically significant differences