



# Restoration of hydroxyindole O-methyltransferase levels in human cancer cells induces a tryptophan-metabolic switch and attenuates cancer progression

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5-Methoxytryptophan (5-MTP) is a tryptophan metabolite with recently discovered anti-inflammatory and tumor-suppressing activities. Its synthesis is catalyzed by a hydroxyindole O-methyltransferase (HIOMT)-like enzyme. However, the exact identity of this HIOMT in human cells remains unclear. Human HIOMT exists in several alternatively spliced isoforms, and we hypothesized that 5-MTP-producing HIOMT is a distinct isoform. Here, we show that human fibroblasts and cancer cells express the HIOMT298 isoform as contrasted with the expression of the HIOMT345 isoform in pineal cells. Sequencing analysis of the cloned isoforms revealed that HIOMT298 is identical to the sequence of a previously reported truncated HIOMT isoform. Of note, HIOMT298 expression was reduced in cancer cells and tissues. Stable transfection of A549 cancer cells with HIOMT298 restored HIOMT expression to normal levels, accompanied by 5-MTP production. Furthermore, HIOMT298 transfection caused a tryptophan-metabolic switch from serotonin to 5-MTP production. To determine the *in vivo* relevance of this alteration, we compared growth and lung metastasis of HIOMT298-transfected A549 cells with those of vector- or untransfected A549 cells as controls in a murine xenograft model. Of note, the HIOMT298-transfected A549 cells exhibited slower growth and lower metastasis than the controls. Our findings provide insight into the crucial role of HIOMT298 in 5-MTP production in cells and in inhibiting cancer progression and highlight the potential therapeutic value of 5-MTP for managing cancer.

5-Methoxytryptophan (5-MTP)<sup>3</sup> is a newly discovered L-tryptophan metabolite possessing anti-inflammatory and tumor-suppressing properties (1, 2). A number of human cell types including fibroblasts and endothelial cells produce and release 5-MTP into the extracellular milieu (1, 2). Molecular genetic and biochemical studies suggest that 5-MTP biosynthesis is catalyzed by tryptophan hydroxylase (TPH) and hydroxyindole O-methyltransferase (HIOMT) (1). TPH-1 is expressed and responsible for 5-MTP synthesis in fibroblasts and endothelial cells (1, 3). However, the exact identity of the HIOMT isoform has not been clearly defined. HIOMT (also known as N-acetylserotonin O-methyltransferase (ASMT)) catalyzes the final step of melatonin synthesis in the pineal gland (4–6). Three transcripts derived from alternative splicing were reported in pineal tissues. The full-length transcript encodes a 373-aa protein (designated HIOMT373). An exon 6-spliced transcript encodes a 345-aa protein (HIOMT345), and an exon 6 and 7-spliced transcript encodes a 298-aa protein (HIOMT298) (Fig. S1) (4). HIOMT345 was reported to be catalytically active for melatonin biosynthesis, whereas HIOMT373 and HIOMT298 were inactive (7). It is unclear whether HIOMT345 or another isoform catalyzes 5-MTP synthesis.

Cancer cells are deficient in 5-MTP production, which contributes to cyclooxygenase-2 (COX-2) overexpression and excessive migration and invasion (1). Addition of chemosynthetic L-5-MTP or coculture with fibroblasts rescues control of COX-2 expression and migratory activity (1, 8). Intraperitoneal administration of 5-MTP to a murine xenograft tumor model attenuates cancer growth and lung metastasis (1). The enzymatic defect responsible for 5-MTP deficiency in cancer cells has not been delineated. We suspected that the defect resides in the HIOMT step. To address this, we analyzed expression of HIOMT isoforms in cancer cells *versus* fibroblasts by RT-PCR

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This article contains Figs. S1–S9 and Table S1.

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<sup>3</sup> The abbreviations used are: 5-MTP, 5-methoxytryptophan; HIOMT, hydroxyindole O-methyltransferase; TPH, tryptophan hydroxylase; COX-2, cyclooxygenase 2; 5-HTP, 5-hydroxytryptophan; ASMT, N-acetylserotonin methyltransferase; AADC, aromatic amino acid decarboxylase; HAT, histone acetyltransferase; aa, amino acid; qPCR, quantitative PCR; Fb, fibroblast(s); nt, nucleotide; PMA, phorbol 12-myristate 13-acetate; SCID, severe combined immunodeficient; UPLC, ultra-performance LC.

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and qPCR and determined HIOMT expression in human cancer tissues by immunohistochemistry. We show that fibroblasts express functional HIOMT298, which is reduced in cancer cells. Cancer cells are unable to convert 5-HTP to 5-MTP but convert 5-HTP to serotonin via aromatic amino acid decarboxylase (AADC). Stable transfection of A549 cancer cells with HIOMT298 restores HIOMT expression accompanied by reduced AADC expression and a switch from serotonin to 5-MTP production. HIOMT298-transfected A549 cells exhibit slower growth and lower metastasis than control A549 cells when implanted into the xenograft model.

### Results

#### Human fibroblasts and cancer cells express HIOMT298 isoform

HIOMT transcripts expressed in A549 and fibroblasts (Fb) *versus* Y79 retinoblastoma cells were analyzed by RT-PCR. To ascertain the identity of HIOMT isoform transcripts on gel, we transiently transfected A549 and Hs68 Fb with HIOMT373, -345, and -298 expression vectors. HIOMT isoform transcripts amplified by RT-PCR were expected to generate products of 397, 313, and 172 bp in length, respectively (Fig. S1). Overexpressed HIOMT isoforms were detected at the expected sizes (Fig. 1A). HIOMT345 isoform band was detected in Y79 cells, whereas isoform 298 was dimly detected in Hs68 fibroblasts and undetected in A549 cells (Fig. 1A). At 40 cycle of PCR amplification, isoform 298 was detected in A549 as well as in Hs68 and 293 T cells, whereas multiple bands were detected in Y79 cells with isoform 345 being predominant (Fig. 1B). The HIOMT mRNA level quantified by qPCR was extremely low in cancer cells when compared with the level in Y79 cells (Fig. 1C).

To determine whether HIOMT isoforms expressed in Fb and A549 cells are identical to those reported for pineal HIOMT (ASMT) isoforms, we cloned HIOMT cDNAs and sequenced them. A vast majority of the clones derived from Fb and A549 had sequence identity to the reported ASMT298 (Fig. 1D and Fig. S2A). Two A549 clones contain an additional 184-nucleotide insert in isoform 373 (designated 373v) and isoform 345 (designated 345v), respectively, and one Fb clone contains the 184-nt insert in isoform 345 (Fig. 1D). The 184-nt sequence is identical to that of an intron 7 fragment (Fig. S2B). These results indicate that the predominant HIOMT isoform expressed in nonpineal cells is isoform 298.

#### HIOMT expression is reduced in human cancer tissues

HIOMT protein expression in cancer *versus* normal cells was analyzed by immunohistochemistry using an HIOMT antibody that detects HIOMT298 as described under "Experimental procedures." Normal colon tissues stained strongly for HIOMT, whereas colon cancer cells exhibited weaker staining (Fig. 2A). Three of 15 (20%) pathological Grade I-II colon cancer samples were strongly stained for HIOMT, whereas none of the Grade III colon cancer samples were strongly stained (Fig. 2A, lower panel). Normal breast tissues were not as strongly stained as normal colon or pancreatic tissues. However, HIOMT staining was weaker in breast cancer than in normal, especially in Grade III breast cancer tissues (Fig. 2B). All normal pancreatic tissues were strongly stained for HIOMT. The staining was reduced in pancreatic cancer in a pathological grade-dependent manner

(Fig. 2C). A small proportion (11%) of Grade I-II pancreatic cancer was strongly stained, whereas none of the Grade III pancreatic cancer exhibited strong staining (Fig. 2C, lower panel). Taken together, these results suggest that cancer cells have reduced expression of HIOMT298. The expression level is correlated with pathological grade of cancer and is heterogeneous, especially in lower grade cancer.

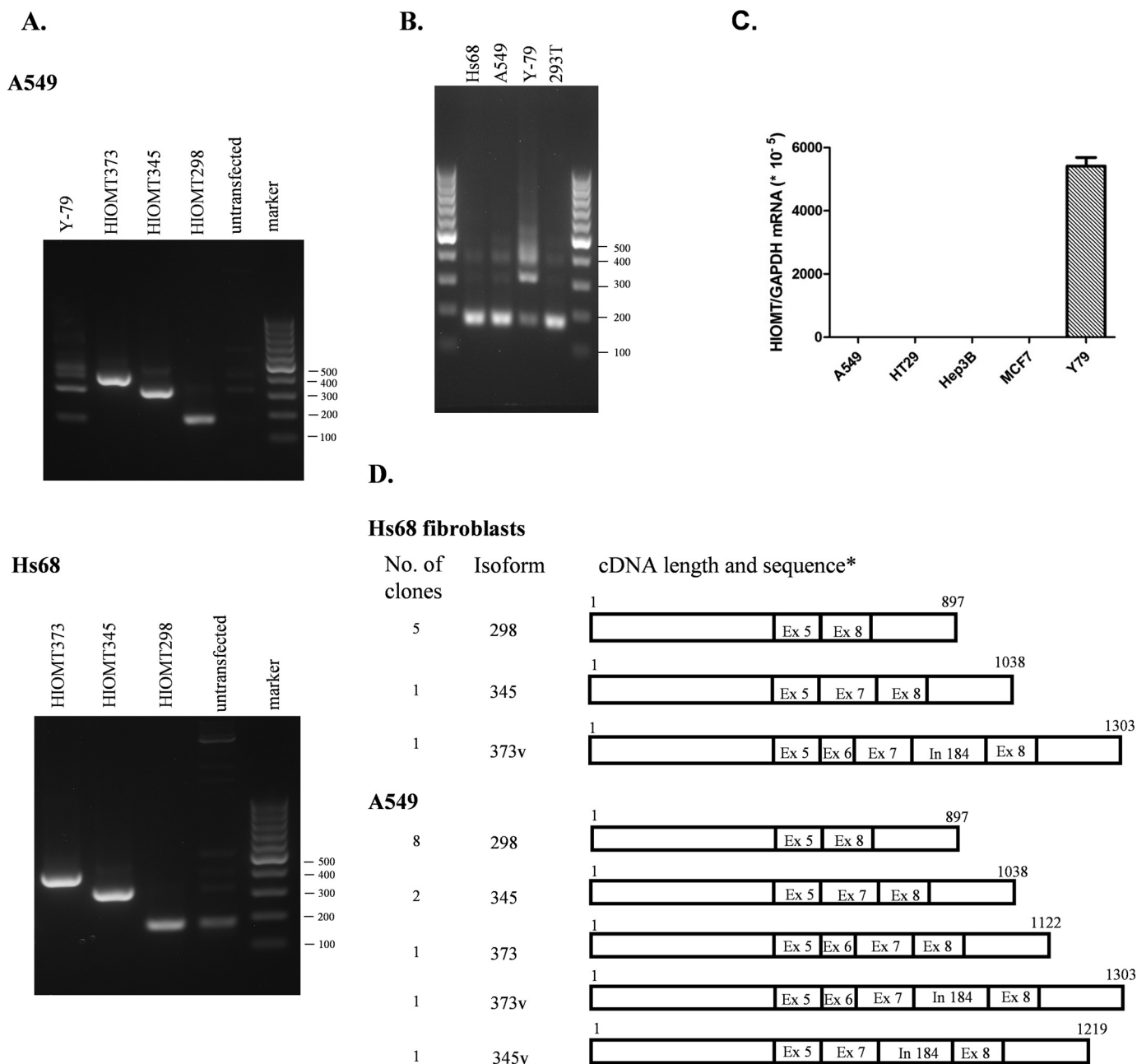
#### Cancer cells fail to convert 5-HTP to 5-MTP and are unable to utilize 5-HTP to control COX-2 expression and p300 activation

Because HIOMT is pivotal in converting 5-HTP to 5-MTP, we evaluated the influence of exogenous 5-HTP on 5-MTP production in cancer cells *versus* Fb. We silenced TPH-1 in Hs68 Fb with selective TPH-1 siRNAs and treated TPH-1-silenced Fb with 5-HTP. As TPH-1 silencing reduces 5-HTP production, we anticipated that exogenous 5-HTP would be converted to 5-MTP via HIOMT. 5-MTP released into the medium was measured by competitive ELISA. These results suggest that A549 cancer cells fail to convert 5-HTP to 5-MTP due to deficient HIOMT expression.

We next determined whether 5-HTP exerts an effect on cancer cell COX-2 expression. We have previously shown that 5-HTP inhibits PMA-induced COX-2 expression in fibroblasts via 5-MTP release but has no effect on COX-2 expression in A549 cells (1). Addition of 5-HTP to other cancer cells, *i.e.* Hep3B, MCF7, and HT1299, also did not suppress PMA-induced COX-2 expression (Fig. 3B), consistent with defective HIOMT activity. Transcriptional coactivator p300 plays a crucial role in COX-2 transcription induced by phorbol esters and proinflammatory cytokines (9, 10). We reported that p300 histone acetyltransferase (HAT) activity is suppressed by 5-MTP in proliferative fibroblasts, leading to reduced COX-2 expression (11). Furthermore, addition of 5-MTP to quiescent fibroblasts results in reduction of p300 HAT activation and COX-2 expression. We determined here whether 5-HTP controls PMA-induced p300 HAT activation in Hs68 fibroblasts *versus* A549 cells. 5-HTP inhibited Fb p300 HAT activation in a concentration-dependent manner but failed to inhibit p300 HAT activation in A549 cells (Fig. 3C). These results provide additional evidence for an HIOMT defect in cancer cells.

#### Stable transfection of cancer cells with HIOMT298 switches from serotonin to 5-MTP biosynthesis

To ascertain restoration of HIOMT expression in A549 cells stably transfected with HIOMT298, we analyzed HIOMT mRNA by real-time qPCR. Basal HIOMT mRNA was very low but was highly increased by HIOMT298 transfection (Fig. 4A). HIOMT298 proteins were undetectable in A549 or FLAG-transfected A549 but were detected in HIOMT298 stable A549 cells (Fig. 4B). We next measured by quantitative LC-MS key bioactive metabolites of 5-HTP released from cells stably transfected with HIOMT298 *versus* controls. A high level of serotonin and very low levels of 5-MTP and melatonin were detected in the medium of untransfected A549 cells (Fig. 4C). The 5-MTP level was increased by ~30-fold, and serotonin was reduced by >10-fold by HIOMT298 transfection. Neither 5-MTP nor serotonin was altered by vector transfection (Fig. 4C). 5-HTP and tryptophan levels were not significantly



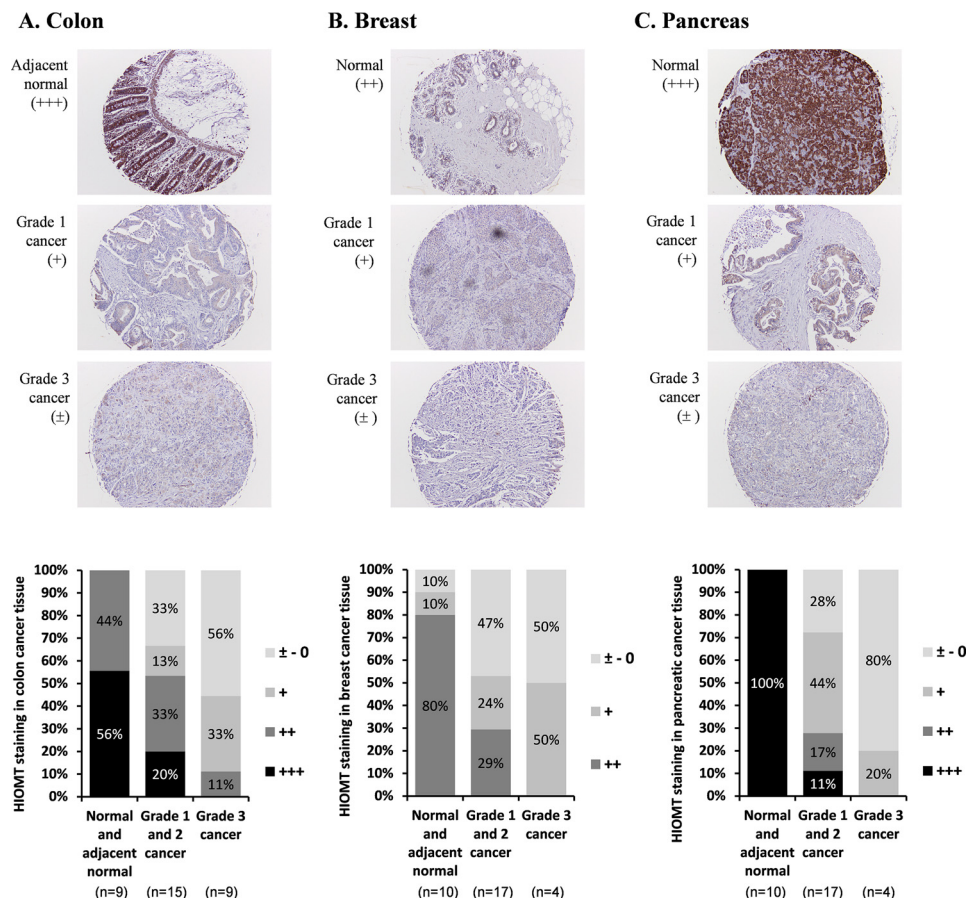
\* Sequence is shown in Figure S2.

**Figure 1. Analysis of HIOMT mRNA in human lung cancer A549 cells, Hs68 fibroblasts, and Y79 cells.** A, A549 and Hs68 were transiently transfected with HIOMT isoform 373, 345, or 298 using transfection reagent Lipofectamine 2000. RNA from transfected and untransfected cells was extracted, and cDNA was reverse transcribed. HIOMT transcript was PCR-amplified for 35 cycles using primer set F2/R5. PCR products were separated by 1.5% agarose gel. The *upper panel* (A549) is a representative of four independent experiments, and the *lower panel* is a representative of two experiments. B, HIOMT mRNA of untransfected cells was PCR-amplified for 40 cycles. This figure is a representative of three independent experiments. C, HIOMT mRNA in the indicated cancer cells was quantified by qPCR. The *error bar* denotes mean  $\pm$  S.E. ( $n = 5$  for Y79;  $n = 4$  each for A549, Hep3B, and MCF7; and  $n = 3$  for HT29). D, cloning and sequencing of Hs68 and A549 transcripts. cDNA length and exon structure are illustrated as a double-strand box. 373v and 345v denote variant clones with an additional 184-nt intron fragment. The detailed sequence of each clone is shown in Fig. S2.

changed by HIOMT298 transfection (Fig. S3). For comparison, we prepared A549 cells stably transfected with HIOMT373 and HIOMT345 isoforms and analyzed HIOMT expression and 5-HTP metabolites. HIOMT isoform proteins and mRNA were detected by Western blotting and RT-PCR, respectively. Their levels of expression were significantly higher than the basal level (Fig. 4, A and B, lower panel). HIOMT345 overexpression did not increase 5-MTP or reduce serotonin (Fig. 4C). Surpris-

ingly, HIOMT373 overexpression changed the 5-MTP/serotonin profile in a manner comparable with HIOMT298 overexpression (Fig. 4C). Overexpression of either isoform failed to influence the melatonin (Fig. 4C) or 5-HTP level (Fig. S3). These results indicate that restoration of HIOMT298 expression in A549 cells switches 5-HTP conversion from serotonin to 5-MTP. Furthermore, the metabolite data suggest that isoform 373 may be catalytically active in 5-MTP synthesis.

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**Figure 2. Reduction of HIOMT expression in human cancer tissues.** Colon, breast, and pancreatic cancer tissues from a cancer tissue array were analyzed by immunohistochemistry using antibody ab180511. Intensity of staining was graded visually as 0, +, ++, and +++. A, colon cancer tissues ( $n = 24$ ). B, breast cancer tissues ( $n = 21$ ). C, pancreatic cancer tissues ( $n = 21$ ). Upper panels show representative staining pattern and intensity, and the lower panel shows the percentage of normal versus cancer tissues with different staining intensity. The HIOMT staining was performed twice with similar results.

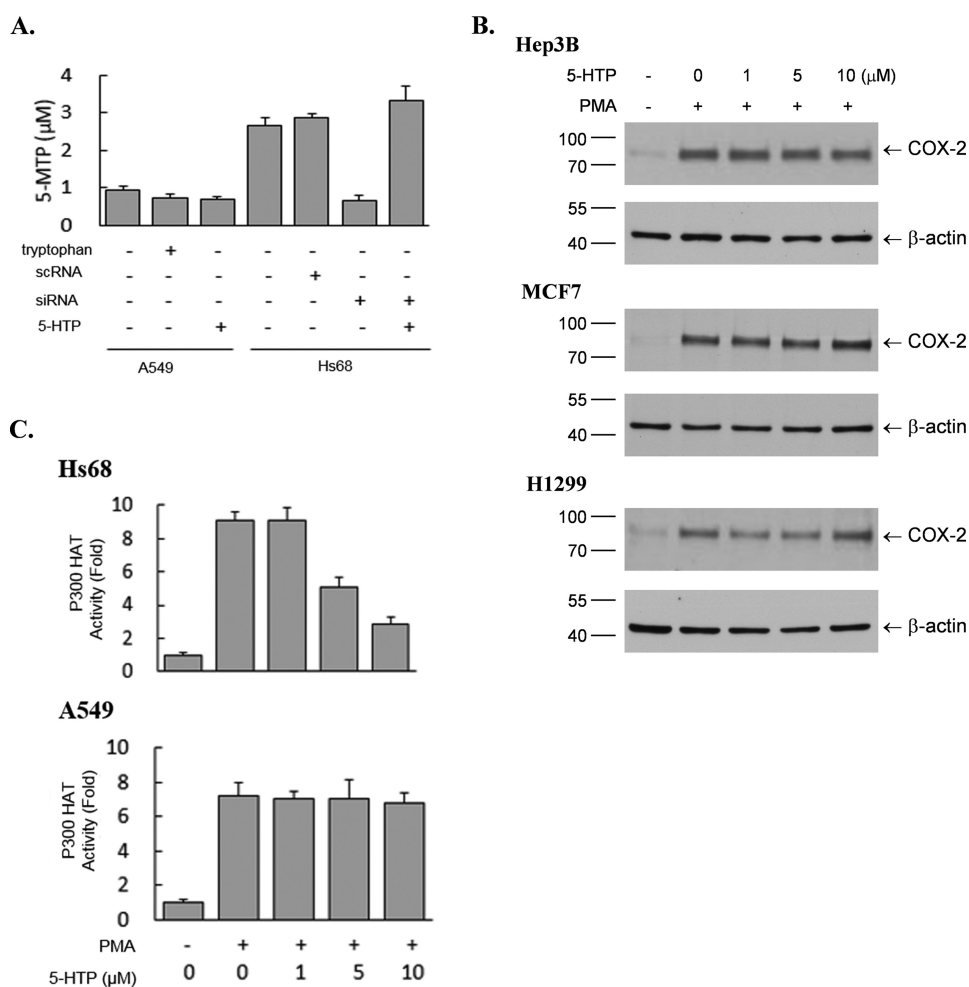
### HIOMT298 transfection suppresses aromatic amino acid decarboxylase expression in A549 cells

Serotonin biosynthesis from L-tryptophan is catalyzed by two enzymatic steps: 1) TPH catalyzes the conversion of L-tryptophan to 5-HTP, and 2) AADC (also known as L-3,4-dihydroxyphenylalanine decarboxylase) converts 5-HTP to 5-hydroxytryptamine (serotonin) (12, 13). We analyzed TPH isoforms and AADC expression by RT-PCR. TPH-1, but not TPH-2, transcript was detected in A549 and several cancer cells, whereas both TPH-1 and TPH-2 transcripts were detected in Y79 cells, Hep3B cancer cells, and WI38 cells, a lung embryonic fibroblast (Fig. S4). AADC mRNA was detected in A549 cells but not in Hs68 human fibroblasts (Fig. S5). To determine whether HIOMT298 transfection influences AADC expression and thereby serotonin release, we measured the AADC mRNA level by qPCR. AADC expression in A549 cells was significantly reduced by HIOMT298 transfection (Fig. 4D). These results suggest that forced HIOMT298 expression in A549 cancer cells results in silencing AADC expression, thereby shunting 5-HTP into the HIOMT pathway.

### A549 cells stably transfected with HIOMT298 grow slower and metastasize less than control A549 cells

We previously observed that intraperitoneal administration of 5-MTP to mice significantly reduced A549-implanted

subcutaneous tumor growth and lung metastasis in a xenograft tumor model (1). Because A549 stably transfected with HIOMT298 restored 5-MTP production, we were curious whether the HIOMT298-corrected cancer cells exhibit reduced growth and metastasis. Untransfected, vector-, and HIOMT298-transfected A549 cells ( $5 \times 10^6$  cells each) were implanted into the subcutaneous tissues of SCID mice. Subcutaneous tumor volume was measured twice weekly by caliper for 7 weeks at which time mice were sacrificed. The subcutaneous tumors and lung tissues were resected. Lung nodules were counted and examined. Tumor growth was similar among the three groups in the initial 18 days (Fig. 5A). By day 22, the tumor volume of the HIOMT298 group was significantly smaller than that of the controls, and by week 7 the mean tumor volume of the HIOMT298 group was <50% of the untransfected control (Fig. 5A). Tumor volume of the FLAG-vector control group was slightly lower than the untransfected A549 control group, but the difference was not statistically significant, whereas the tumor volume of the HIOMT298 group was significantly lower than that of the FLAG vector control group (Fig. 5A). Resected tumors in the HIOMT298 group were smaller than those in the untransfected A549 or FLAG-transfected group (Fig. 5B). Metastatic lung nodules were also reduced in the HIOMT298-transfected A549 group when compared with the A549 or A549-FLAG control group (Fig. 5C). Body weight of the



**Figure 3. Cancer cells fail to convert 5-HTP to 5-MTP.** A, A549 cells were incubated with 5-HTP (10 μM) for 4 h, and 5-MTP in the medium was measured by competitive ELISA. For Hs68 fibroblasts, TPH-1 was silenced with siRNA, and TPH-1-silenced cells were incubated with 5-HTP (10 μM). 5-MTP released into the medium was measured by ELISA. B, several types of cancer cells were incubated with different concentrations of 5-HTP for 4 h, and COX-2 was analyzed by Western blotting. C, Hs68 (upper panel) or A549 (lower panel) cells were pretreated with 5-HTP for 30 min followed by PMA (100 nM) for 4 h. p300 in the cell lysates was isolated by immunoprecipitation, and its HAT activity was analyzed. Error bars in A and C denote mean ± S.E. (n = 3). scRNA, small control RNA.

HIOMT298 group was not different from that of control groups (Fig. S6A). The *in vivo* animal data support the concept that correction of the HIOMT defect in A549 cells alters cell metabolism and phenotype and converts them into a less aggressive cancer with reduced migration and metastasis.

#### A549-HIOMT298 xenograft tumor tissues express a higher HIOMT transcript level than control tissues

To determine whether decline of tumor volume and lung metastasis of HIOMT298-transfected A549 cells is correlated with HIOMT expression, we isolated RNA from the resected tumors and analyzed HIOMT mRNA by qPCR. Tumors derived from A549 and A549-FLAG expressed a low level of HIOMT transcript. HIOMT transcript in tumors derived from A549-HIOMT298 was 7-fold higher than that in control tumors (Fig. 5D).

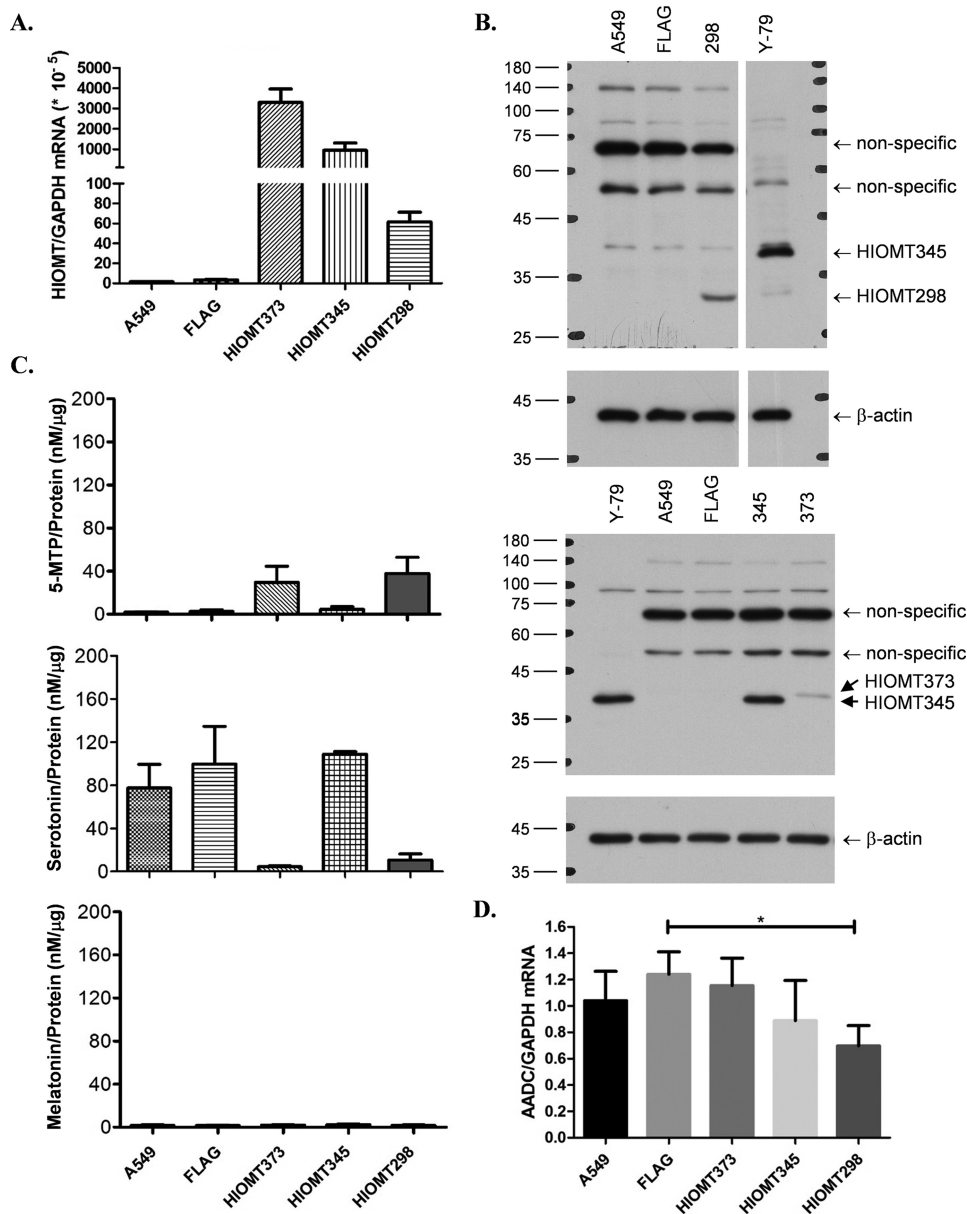
#### 5-HTP administration does not enhance the antitumor action of HIOMT298-A549 cells

As 5-HTP occupies a central position in 5-MTP synthesis, we determined whether intratumor administration of 5-HTP provides additional beneficial effect on reducing tumor growth and

metastasis. The control (A549 + PBS) group had a significantly larger tumor volume than the HIOMT298 + PBS group (Fig. 6A). 5-HTP administration to HIOMT298-A549 resulted in a slight reduction of tumor volume at week 7, but the difference was not statistically significant (Fig. 6A). Resected tumors were smaller in HIOMT298 and HIOMT298 + 5-HTP groups as compared with those in the A549 control (Fig. 6B). Two mice in the 5-HTP + HIOMT298-A549 group had lung metastasis, whereas no metastasis was noted in the HIOMT298-A549 without 5-HTP group (Fig. 6C). 5-HTP administration did not significantly influence body weight (Fig. S6B). HIOMT mRNA levels in tumor tissues were increased by HIOMT298 transfection but were not influenced by 5-HTP administration (Fig. 6D). Taken together, these results indicate that A549 lung cancer growth and metastasis in the xenograft mouse model are reduced by restoring HIOMT expression, and addition of 5-HTP does not enhance the beneficial effect.

#### Discussion

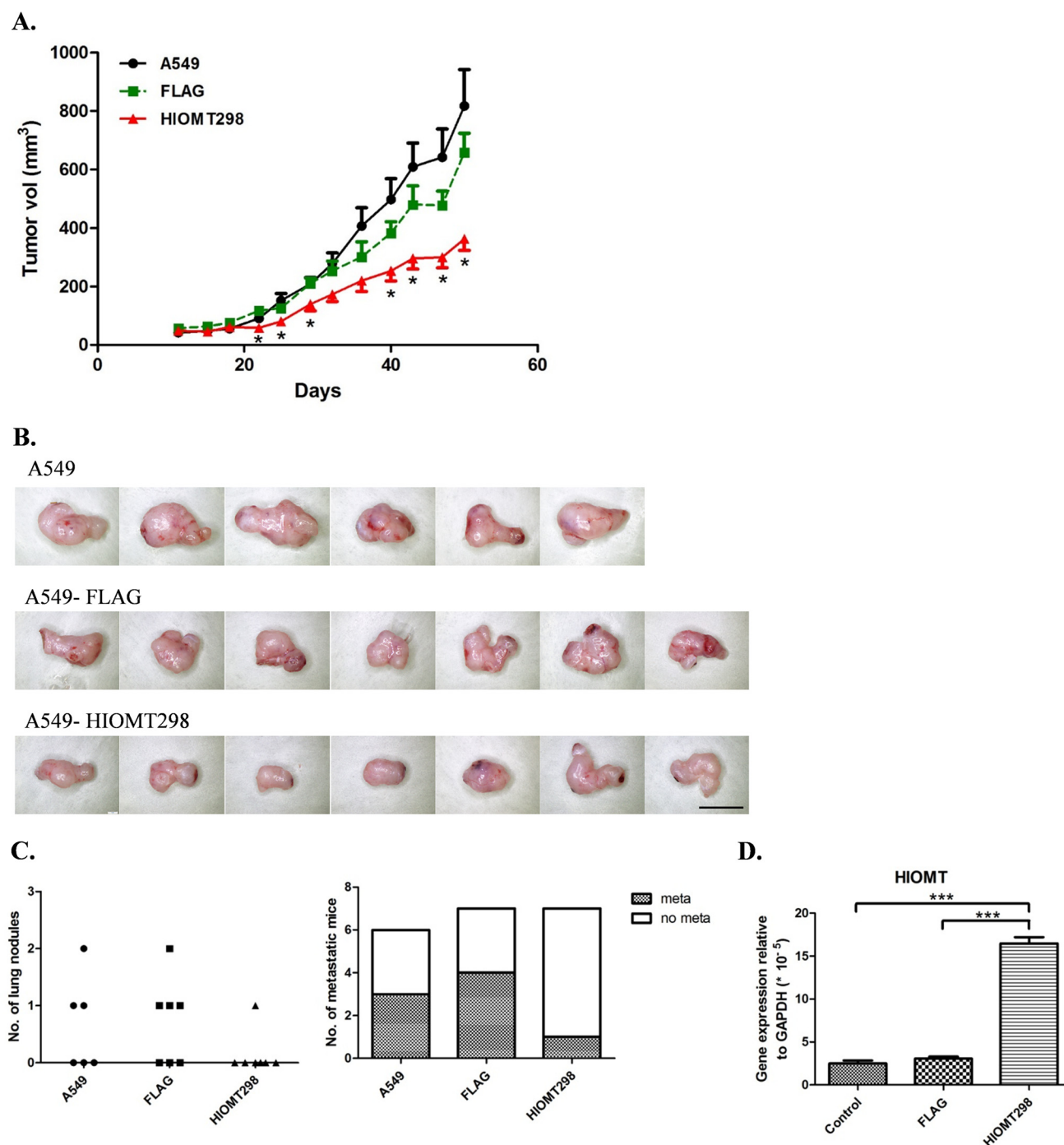
Different cell types in mammals catabolize L-tryptophan into distinct bioactive metabolites through the expression of specific enzymatic pathways. Several metabolites including kynu-



**Figure 4. A549 cells stably transfected with HIOMT regain 5-MTP production accompanied by reduction of serotonin biosynthesis.** *A*, HIOMT mRNA in untransfected A549 cells, vector-transfected A549 cells (FLAG), and A549 stably transfected with HIOMT373, -345, and -298 isoforms. HIOMT isoform mRNA was measured by qPCR using GAPDH as reference. Each bar denotes mean  $\pm$  S.E. ( $n = 3$ ). *B*, Western blot analysis of HIOMT in A549 cells with and without stable transfection with HIOMT isoforms. The upper panel shows A549 cells stably transfected with the 298 isoform versus untransfected A549 and FLAG control. Y79 cells without transfection were included as reference. The lower panel shows A549 cells stably transfected with HIOMT345 and -373 isoforms. As indicated by arrows, several nonspecific bands were detected by this antibody. *C*, major 5-HTP metabolites including 5-MTP, serotonin, and melatonin were quantified by UPLC-MS using multiple reaction monitoring mode. For each metabolite, a calibration curve was established. The error bars denote mean  $\pm$  S.E. ( $n = 3$ ). *D*, AADC mRNA in untransfected A549 cells ( $n = 5$ ), FLAG vector ( $n = 5$ ), HIOMT298-transfected A549 cells ( $n = 5$ ), and isoform 373- and 345-transfected A549 ( $n = 3$  each) was analyzed by RT-PCR. Error bars in *A* and *D* represent mean  $\pm$  S.E. \* denotes  $p < 0.05$ .

renine, serotonin, melatonin, and cytoguardin (5-MTP) modulate cancer cell proliferative and migratory activities in opposite directions. For example, among metabolites derived from the 5-HTP pathway, serotonin promotes cancer growth (14–17), whereas melatonin and cytoguardin suppress tumorigenesis (18–20). Although it is well known that serotonin is primarily produced by serotonergic neurons, melatonin by cells in the pineal gland, and cytoguardin by fibroblasts and endothelial cells, it is unclear whether cancer cells possess enzymatic machinery to synthesize any of those bioactive metabolites. In this study, we provide novel information to advance the under-

standing of how a cancer cell utilizes L-tryptophan metabolism to promote its growth and migration. Our results indicate that cancer cells express TPH-1 and AADC and release abundant serotonin into the extracellular milieu but are defective in HIOMT expression and hence unable to produce antitumor metabolites. Thus, serotonin acts in a paracrine manner to promote cancer growth without opposition from cytoguardin or melatonin. Importantly, our results show that cancer cells can be engineered to switch to generate 5-MTP by forced expression of HIOMT isoform 298. Overexpression of HIOMT298 switches 5-HTP catabolism from serotonin to 5-MTP.



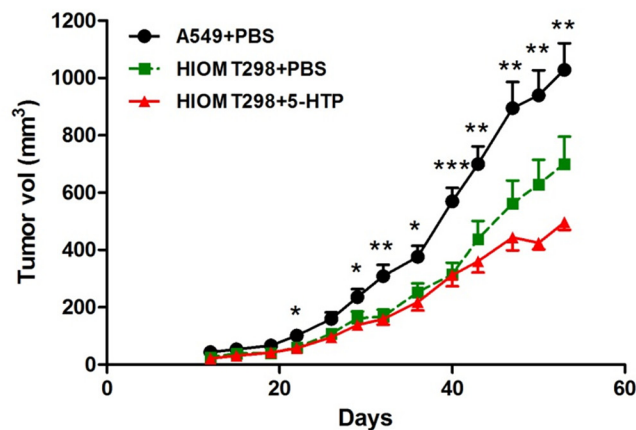
**Figure 5. Analysis of tumor volume and lung metastasis of A549 versus A549 HIOMT298 implantation in a murine xenograft tumor model.** A549 ( $5 \times 10^6$  cells) with or without HIOMT298 transfection and vector-FLAG-transfected A549 cells were implanted subcutaneously into SCID mice. *A*, tumor volume was evaluated by caliper measurement twice weekly for 7 weeks. Error bars represent mean tumor volume  $\pm$  S.E. Tumor volume in the untransfected A549 group ( $n = 6$ ) or vector group ( $n = 7$ ) was significantly higher than in the HIOMT298-transfected A549 group ( $n = 7$ ). \* denotes  $p < 0.05$  compared with control groups. There was no significant difference between the untransfected and the vector groups. *B*, resected tumors from each animal. Scale bar, 10 mm. *C*, lung metastasis. The left panel shows a scatter graph of the number of nodules. Each point represents a single mouse. The right panel shows the number of animals with lung nodules. *D*, HIOMT mRNA in resected tumors was analyzed by qPCR. Error bars represent mean  $\pm$  S.E. ( $n = 3$ ). \*\*\* denotes  $p < 0.001$ .

HIOMT298-overexpressing A549 cells had slower growth and fewer lung metastatic nodules than control A549 cells in the xenograft model. Changes in A549 phenotype by HIOMT298 overexpression are attributable to increased 5-MTP production and/or reduced serotonin production. We previously reported that intraperitoneal administration of 5-MTP reduces tumor volume and lung metastasis in an A549 xenograft model

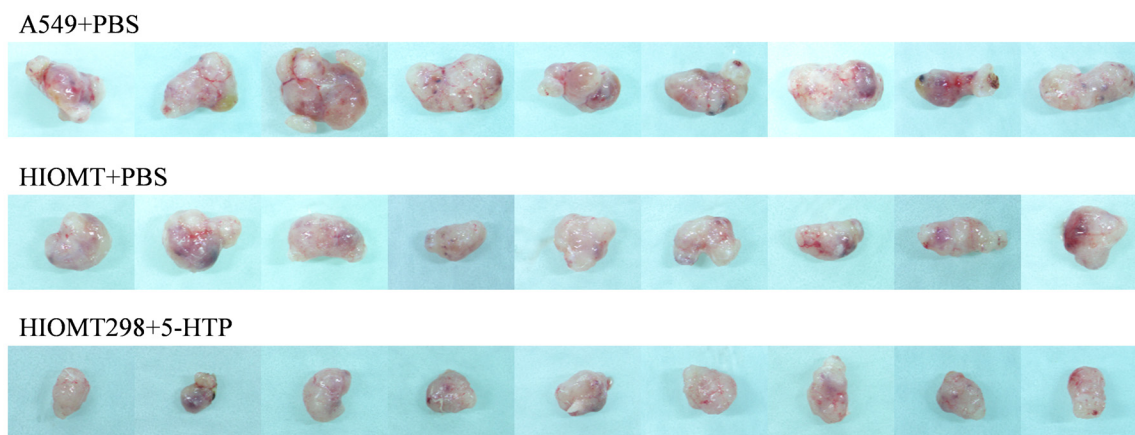
(1). Thus, increased 5-MTP production by HIOMT298-overexpressing A549 cells contributes to self-control of tumor growth. It is less clear whether reduction of serotonin production in HIOMT298-overexpressing A549 cells directly influences tumor growth and metastasis. Further studies are needed to evaluate this. Taken together, our findings suggest that cancer cells such as A549 cells produce predominantly serotonin

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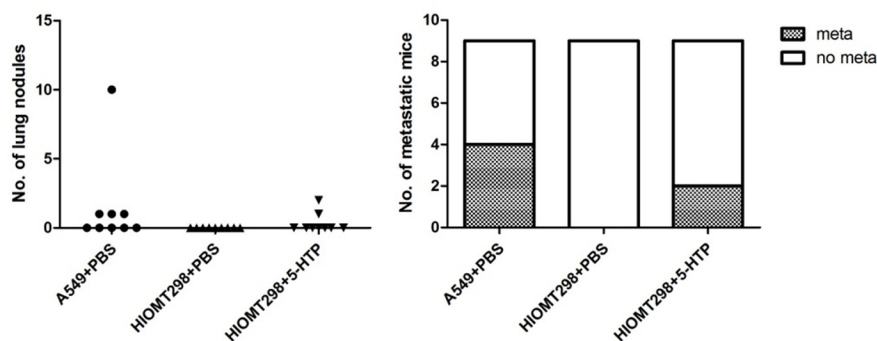
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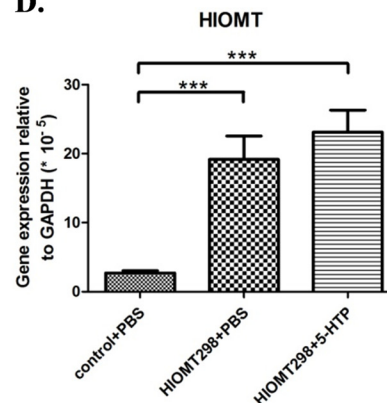
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C.



D.



**Figure 6. Influence of 5-HTP on tumor growth in the xenograft model.** A549 cells without transfection or with HIOMT298 transfection were implanted into SCID mice (nine mice per group). A, tumor volume was measured twice weekly for 7 weeks. Error bars represent mean  $\pm$  S.E. \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ , and \*\*\* denotes  $p < 0.001$  compared with HIOMT298 + PBS group. The difference between HIOMT298 + 5-HTP and HIOMT298 + PBS was not statistically significant. B, resected tumors on day 54 from each mouse of each group. Scale bar, 10 mm. C, number of lung nodules (left panel) and number of mice with lung nodules in each group (right panel). D, HIOMT mRNA levels in tumor tissues were analyzed by qPCR. Error bars represent mean  $\pm$  S.E. ( $n = 3$ ). \*\*\* denotes  $p < 0.001$ .

due to HIOMT silencing and default expression of AADC, and HIOMT298 transfection results in restoration of HIOMT298, which suppresses AADC expression and induces a metabolic switch to 5-MTP production (Fig. S7). We propose that 5-MTP acts in an autocrine or paracrine manner to subdue cancer cell proliferation and migration. It is also possible that 5-MTP may

act on stromal cells in the tumor microenvironment to protect stromal cells from cancer-induced phenotypic changes.

Lung cancer cells were reported to constitutively express AADC (21). Two distinct AADC mRNAs due to alternative splicing were reported, and A549 cells express predominantly the nonneuronal mRNA type (21). AADC mRNA levels are



increased in several types of cancer including lung and prostate cancer (22). However, little is known about AADC protein expression and its catalytic activities. Nor is it clear how AADC expression is transcriptionally regulated. Our findings shed light on the control of AADC expression by HIOMT/5-MTP. HIOMT298 overexpression in A549 cells exerts a strong suppressive effect on AADC expression, probably through 5-MTP. 5-MTP is known to inhibit expression of COX-2 and diverse proinflammatory cytokines by blocking p300 HAT activation and the activities of NF- $\kappa$ B, CCAAT/enhancer-binding protein  $\beta$ , and AP-1 (11). It was reported that AADC expression depends on hepatocyte nuclear factor-1 (23). It is unclear whether 5-MTP inhibits hepatocyte nuclear factor-1 binding to AADC via p300 HAT. Work is in progress to address this question.

Our data reveal the heterogeneity of HIOMT expression in human colon, breast, and pancreatic cancer tissues. A majority of each of the three cancer tissues express a low level of HIOMT, but a fraction of them express a normal or intermediate level of HIOMT (Fig. 2). It remains to be determined whether the cancer tissue HIOMT level correlates with clinical cancer progression and/or response to therapy. We have recently reported detection of 5-MTP in human blood: mean serum 5-MTP concentration of 30 healthy subjects was  $\sim 1 \mu\text{M}$  (2). Serum 5-MTP concentrations in cancer patients have not been reported. It will be of value to correlate serum 5-MTP with tissue HIOMT and relate this correlation to cancer progression and treatment outcome.

Our results show that A549 cells stably transfected with HIOMT298 regain 5-MTP secretion, suggesting that this truncated HIOMT is catalytically active in 5-MTP synthesis. By contrast, stable transfection of A549 cells with the HIOMT345 isoform has no effect on 5-MTP synthesis. These results are strikingly different from those reported in pineal cells that show that the ASMT (HIOMT) 345 isoform is active whereas the 298 isoform is inactive in melatonin generation (7). X-ray structural analysis of ASMT345 has revealed that the binding site of the methyl group donor, *S*-adenosyl-*L*-methionine (SAM), is adjacent to the binding site of the substrate, *N*-acetylserotonin (7). As exon 7 segment codes for the peptide sequence that lines the SAM-binding site, isoform 298 in which exon 7 is spliced was predicted to have a weaker binding for SAM than isoform 345. Because SAM binding may be crucial for 5-MTP synthesis, it is intriguing that isoform 298 is active in catalyzing 5-MTP biosynthesis. It is surprising that A549 cells stably transfected with the 373 isoform release 5-MTP into the extracellular milieu to an extent comparable with A549 cells transfected with the 298 isoform, suggesting that HIOMT373 isoform is also active in catalyzing 5-MTP synthesis. The 373 isoform contains an extra exon 6, which codes for a segment that structurally blocks SAM binding (7). The reason why the 298 and 373 isoforms are catalytically active in 5-MTP synthesis despite their defects in SAM binding is unclear. We speculate that a different methyl donor may be used in 5-MTP synthesis. Further structure–function analysis of 5-MTP *versus* melatonin synthesis will be needed to resolve this paradox.

5-HTP production from *L*-tryptophan via TPH is a limiting step in serotonin and melatonin biosynthesis. We therefore

determined in the xenograft tumor model whether administration of 5-HTP would produce additional benefit in reduction of tumor volume and metastasis. Our findings indicate that administration of 5-HTP does not significantly enhance the effect of HIOMT298 overexpression, suggesting that 5-HTP is not a limiting factor in 5-MTP production and that its administration does not have additional beneficial effects.

Results from this study imply that HIOMT298 could serve as a theranostic biomarker for chemoprevention and treatment of cancer with 5-MTP or its synthetic analogs. 5-MTP and synthetic analogs will be well suited for chemoprevention of certain types of human cancer because 5-MTP is endogenously produced and expected to have predictable toxicity. Furthermore, 5-MTP is highly effective in controlling an array of proinflammatory mediators including cytokines, chemokines, and COX-2 (2) and hence has potential to control the inflammatory microenvironment and limit cancer growth and metastasis.

## Experimental procedures

### Cell culture

Human A549, 293T, HT29, MCF7, Hep3B, H1299, and WI38 cells were obtained from American Type Culture Collection (ATCC). Except for HT29, they were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> incubator. HT29 cells were maintained in 90% McCoy's 5A medium supplemented with 10% FBS. Hs68 fibroblasts were obtained from ATCC and cultured as described previously (1). Y79 cells were obtained from the Bioresource Collection and Research Center, Hsin-Chu, Taiwan and cultured in 90% RPMI 1640 medium (ATCC modification) supplemented with 10% FBS. All cultured cells tested negative for mycoplasma contamination.

### Materials

5-HTP was obtained from Sigma-Aldrich. *L*-5-MTP was custom-synthesized by AstaTech (Bristol, PA). Purity of 5-MTP was verified by LC-MS (2). 50 mM *L*-5-MTP was prepared in DMSO and stored at  $-20$  or  $-80$  °C. For cellular experiments, the stock solution was diluted to the indicated concentrations. The final DMSO concentration was  $<0.1\%$ .

### Western blot analysis

HIOMT proteins were analyzed by Western blotting as described previously (1). We evaluated several HIOMT antibodies from different commercial sources and found an antibody from Abcam (catalog number ab180511) to recognize all three isoforms. ab180511 is a rabbit monospecific IgG antibody against human ASMT (HIOMT). For Western blot analysis, we used the antibody at 1  $\mu\text{g/ml}$  concentration. We included Y79 retinoblastoma cell line as a positive control for HIOMT345. To provide evidence for detection of the HIOMT298 isoform, we transfected A549 cells with increasing concentrations of HIOMT expression vectors (0.625–5  $\mu\text{g}$ ) and analyzed the expressed protein levels in cell lysates. An  $\sim 33$ -kDa band consistent with HIOMT298 was detected in the HIOMT298-transfected A549 cell lysates in a concentration-dependent manner (Fig. S8). By contrast, a large molecular mass band at  $\sim 39$  kDa

## Cancer cell HIOMT deficiency

was detected in Y79 cell lysates, consistent with expression of HIOMT345. It is to be noted that multiple nonspecific bands were detected by the antibodies (Fig. S8). To confirm that the antibodies detect HIOMT298, we transiently transfected HIOMT298 stably transfected A549 cells with several different HIOMT siRNAs and control siRNA and analyzed HIOMT298 protein expression. HIOMT298 proteins were undetectable in siRNA-treated cells and remained detectable in cells treated with control siRNA (Fig. S9). Thus, this antibody detects HIOMT298 and was used throughout the experiments. COX-2 proteins were analyzed by Western blotting using polyclonal rabbit anti-COX-2 antibodies obtained from Cell Signaling Technology (catalog number 4842). COX-2 antibodies were used at 1:1000 dilution.

### Analysis of p300 HAT activity

The assay was performed as described previously using a HAT enzyme immunoassay kit (11).

### Measurement of 5-MTP by enzyme immunoassay

5-MTP was measured by a competitive ELISA as described previously (2). Polyclonal rabbit anti-5-MTP antibodies were obtained from Abcam. For each experiment, a calibration curve was established by using pure 5-MTP (0.01–50  $\mu\text{M}$ ) as standards.

### Suppression of TPH-1 with siRNA

Knockdown of TPH-1 expression in Hs68 fibroblasts or HIOMT298 in A549 cells was performed as described previously (1). In brief, Hs68 fibroblasts were transfected with a mixture of three TPH-1 siRNAs and a control RNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). TPH-1 suppression was verified by Western blotting (1).

### HIOMT siRNA transfection analysis

$2.5 \times 10^5$  HIOMT298 stably transfected A549 cells were seeded on a 6-well plate the day before transfection. 4  $\mu\text{l}$  of 20  $\mu\text{M}$  HIOMT or control siRNA was transfected into the cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) for 3 days. Cell lysates were analyzed by Western blotting using a HIOMT antibody from Abcam (catalog number ab180511). Five different HIOMT siRNAs and two different control siRNAs were used for transfection. siHIOMT-1, -2, and -3 and siControl-1 were obtained from GenePharma (Shanghai, China). siHIOMT-4 (catalog number L-012664-00-0005), siHIOMT-5 (catalog number M-012664-01-0005), and siControl-2 (catalog number D-001810-10-05) were purchased from Dharmacon (Lafayette, CO). The sequences of the siRNAs are as follows: siHIOMT-1, 5'-GCAGCAUGCUGAAGUACAUTT-3'; siHIOMT-2, 5'-GCCUUUGACCUGUCAGUGUTT-3'; siHIOMT-3, 5'-GGCAUUCUGUAAUUGAAATT-3'; siHIOMT-4, pool of four siRNAs including 5'-GGUGGUGGCAUUCUGUAA-3', 5'-CCUACGCCUCCUAAUGA-3', 5'-UGGCAGACGCCGUGAGAGA-3', and 5'-GAAGCGUGCUGACCGCCUU-3'; siHIOMT-5, pool of four siRNAs including 5'-GAGAGGAUCUACCACACUU-3', 5'-GAAGUGGUGUGGACGGCAA-3', 5'-GGAUGUAAGAUCACCGUUU-3', and 5'-UGGCAGACGCCGUGAGAGA-3'; siControl-1, 5'-UUCUCCGAACGUGUCACGUTT-3'; siControl-2, pool of four

siRNAs including 5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUGUGUGA-3', 5'-UGGUUUACAUGUUUCUGA-3', and 5'-UGGUUUACAUGUUUCCUA-3'.

### Measurement of 5-HTP metabolites by ultraperformance LC (UPLC)-MS

Tryptophan metabolites were measured using UPLC coupled with a Xevo<sup>TM</sup> triple quadrupole mass spectrometer (Waters). Liquid chromatography was performed on an Acquity UPLC system (Waters) using a BEH C<sub>18</sub> column (1.7  $\mu\text{m}$ , 2.1  $\times$  100 mm; Waters). UPLC linear gradient conditions were: 0–1.5 min, 1% B; 1.5–2.5 min, 5% B; 2.5–4.5 min, 100% B; 4.5–5.0 min, 100% B; 9.5–12 min, 99% A (solvent system A, water/formic acid (100:0.1, v/v); B, acetonitrile/formic acid (100:0.1, v/v)). The injection volume was 2  $\mu\text{l}$ , and the column temperature was maintained at 35 °C. Mass spectrometry detection was performed using a Xevo triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in positive ionization mode. The online MS analysis was using multiple reaction monitoring mode. Parameters for the cone energy and collision energy for these tryptophan metabolites are listed in Table S1. Quantification of these tryptophan metabolites was done using Target Lynx software (Waters). Calibration curves were established by using pure tryptophan at concentrations of 0.0–4.86  $\mu\text{M}$ , 5-MTP at concentrations of 0.0–4.24  $\mu\text{M}$ , serotonin at concentrations of 0.0–5.66  $\mu\text{M}$ , and melatonin at concentrations of 0.0–4.30  $\mu\text{M}$ , respectively.

### HIOMT gene cloning, plasmid construction, and isoform overexpression

RNA from Hs68 and A549 cells was extracted using an RNeasy Mini kit (Qiagen), and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). HIOMT transcript was amplified by PCR using HIOMT-specific primers corresponding to the coding region of HIOMT cDNA (forward, 5'-ACGGCTGGATTGGAGACA-3'; reverse, 5'-CGT-TAGTCCAGGTCACAAGA-3') using *Pfu* Ultra II Fusion HS DNA polymerase (Stratagene). PCR conditions were set at 95 °C for 1 min followed by 40 cycles at 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 80 s. At the end of the amplification, 0.25  $\mu\text{l}$  of *Ex Taq* DNA polymerase (TaKaRa) was added to the PCR product and incubated at 72 °C for 30 min for A-tailing. Following PCR cleanup using a Clean/Gel Extraction kit (BioKit Biotech), the product was cloned into yT&A cloning vector (Yestern Biotech). The entire sequence of HIOMT subclones was read by AB3730XL Sanger sequencing. HIOMT from yT&A vector was further PCR-amplified by primer HIOMT-ClaF/HIOMT-XbaR with stop codon in the reverse primer (hHIOMT-ClaF, 5'-AATAATCGATAATGGGATCCTCAGAGGAC-3'; hHIOMT-XbaR, 5'-CTCTAGATTATTCCTGGCTAA-3'). The PCR product was digested with ClaI and XbaI and ligated to the ClaI/XbaI fragment of p3xFLAG-CMV-14 (Sigma-Aldrich), named HIOMT/CMV-14. The entire coding sequence was confirmed by autosequencing. HIOMT/CMV-14 constructs were transfected into A549 cells using Lipofectamine 2000.

### Preparation of HIOMT stably transfected A549 cells

A549 cells transfected with HIOMT298, -345, or -373 isoform were selected with 400  $\mu\text{g}/\text{ml}$  G418 in DMEM. cDNAs of stable cell lines were verified by PCR amplification using primers F2/R5 (forward, 5'-AGGAGGTCTGGAGCGTCAAC-3'; reverse, 5'-AGGTGTGAGCACTTCCGTCTG-3'). Vector p3xFLAG-CMV-14 (vector-FLAG) was stably transfected into A549 cells as a control. Expression of the vector was verified by primer CMV30F/CMV24R (CMV30-F, 5'-AATGTCGTAATA-ACCCCGCCCGTTGACGC-3'; CMV24-R, 5'-TATTAGGA-CAAGGCTGGTGGGCAC-3').

### Sequence alignment of HIOMT isoforms

cDNA sequences of HIOMT isoforms 373, 345, and 298 from the subclones on the  $\gamma\text{T}\&\text{A}$  cloning vector were translated to amino acids, and the amino acid sequences were compared with the HIOMT sequences in the database using the MultAlin website ("multiple sequence alignment with hierarchical clustering") (24).

### Analysis of HIOMT isoforms and AADC transcripts by RT-PCR

RNA from cells was extracted, and cDNA was synthesized as described above. To distinguish between different HIOMT isoforms, cDNA was PCR-amplified by *Pfu* Ultra II Fusion HS DNA polymerase using primer F2/R5. AADC transcript was amplified by PCR using the following primers: forward, 5'-GCCATCAGGATTCAGGGCTT-3'; reverse, 5'-GCCCCA-GAATGACTTCCACA-3'. PCR conditions were set at 95 °C for 1 min followed by 35–40 cycles of PCR at 95 °C for 20 s, 57 °C for 20 s, and 72 °C for 45 s. PCR products were separated by 1.5% agarose gel.

### Immunohistochemical staining of HIOMT

The cancer tissue array (BC001128, US Biomax) was immunostained with rabbit anti-HIOMT antibody (ab180511; dilution, 1:10) by the core pathology laboratory service in the National Health Research Institutes. The intensity of the HIOMT staining was semiquantified.

### Xenograft tumor experiments

6-week-old male Fox Chase SCID mice, purchased from BioLASCO, were housed in a daily cycle of 12-h light and 12-h darkness under pathogen-free conditions at the Animal Center of National Health Research Institutes. The animal experiment was performed with protocols approved by the Institutional Animal Care and Use Committee of the National Health Research Institutes. SCID mice were randomly assigned to receive implantation of various types of A549 cells: untransfected A549 cells or A549 stably transfected with HIOMT298 or vector-FLAG with or without 5-HTP injection. Cells were harvested with trypsin, and  $5 \times 10^6$  cells/0.1 ml of PBS were inoculated into the mice subcutaneously. Tumor volume was measured by caliper, and body weight was recorded twice weekly for 7 weeks. In mice receiving intratumor 5-HTP, 100  $\mu\text{l}$  of 5-HTP (100  $\mu\text{M}$ ) was injected into tumors twice weekly. Tumor volume was calculated by the formula of length  $\times$  width  $\times$  width/2. Seven weeks after xenotransplantation, mice

were euthanized with  $\text{CO}_2$  inhalation. The subcutaneous tumor and the lungs were removed. One part of the tumor was fixed with 10% formalin for paraffin embedding, tissue sectioning, and hematoxylin and eosin staining. One part of the tumor was quickly frozen in liquid nitrogen and stored at  $-80$  °C for analysis of HIOMT with qPCR. Lungs were also fixed in formalin, and the metastatic nodules of each lung were carefully examined under a steric microscope and further confirmed by histological examination.

### Quantification of HIOMT mRNA by qPCR

HIOMT298 mRNA in xenograft tumor tissues and A549 cells stably transfected with HIOMT298 was quantified by qPCR. RNA was extracted from tumor tissue or cells, and cDNA was synthesized as described above. HIOMT and GAPDH transcripts were analyzed by the StepOnePlus Real-Time PCR System (Thermo Fisher) using KAPA SYBR FAST Master Mix (2 $\times$ ) ABI Prism (Kapa Biosystems). Primers used in quantifying HIOMT mRNA by real-time PCR are: forward, 5'-TGGTGTCCCAGGTTCTCTTC-3'; reverse, 5'-CTCCCCTCGTCTCCACTTTC-3'. Primers for GAPDH are: forward, 5'-GAGTCAACGGATTTGGTCGT-3'; reverse, hGAPDH-R3, 5'-GCATCGCCCACTTGATTTT-3'. Target gene expression relative to GAPDH was calculated as  $2^{-(\text{Ct of target gene} - \text{Ct of GAPDH})}$ .

### Statistical analysis

For tumor growth curves and tumor tissue mRNAs, the statistical significance was analyzed by one-way analysis of variance followed by Newman–Keuls multiple comparison test.  $p < 0.05$  was considered to be statistically significant. The statistical significance of lung metastasis was analyzed by  $\chi^2$  test.

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