

Patterns of Expression of H₂S-Producing Enzyme in Human Renal Cell Carcinoma Specimens: Potential Avenue for Future Therapeutics

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Abstract. *Background:* Renal cell carcinoma (RCC) is the most common cancer of the kidney. The most common histotype is clear-cell (cc) RCC. Hydrogen sulfide (H₂S) is an angiogenic and anti-apoptotic gasotransmitter that is elevated under pseudohypoxic conditions. H₂S is endogenously produced by three enzymes: Cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST). Seeing as increased expression of these enzymes has been observed in other human cancer types, this study aimed to quantify H₂S-producing enzyme expression in human RCC samples and evaluate whether it correlated with clinical outcomes. *Patients and Methods:* Eighty-eight human kidney tissue specimens, with healthy and cancerous tissue components, were immunohistochemically stained for CSE, CBS, and MPST. The mean pixel intensity of positively stained areas was quantified. A retrospective analysis was conducted to obtain patient demographics, rates of metastasis/recurrence,

and prognostic characteristics. Statistical correlations between enzyme expressions and subsequent patient outcomes were evaluated. *Results:* There was significantly greater expression of CSE, CBS, and MPST in cc-RCC compared to paired healthy tissue ($p < 0.0001$). The difference in expression of CSE in cancerous versus normal tissue was significantly greater than that for CBS and MPST ($p < 0.0001$ and $p < 0.01$, respectively). Enzyme expression patterns in cancerous versus normal tissue did not correlate with nuclear grade, stage, histological type or cancer recurrence/metastasis. *Conclusion:* To our knowledge, this is the first report of the differential increase in expression of CSE, CBS, and MPST in human RCC. Although these patterns do not appear to correlate with cancer recurrence, metastasis, size or nuclear grade, their differential increase suggests a potential therapeutic target.

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Renal cell carcinoma (RCC) is the most common cancer of the kidney. It accounts for 80-90% of all kidney cancer cases and has a cancer-specific mortality rate of 30-40% (1, 2). Incidence rates of RCC have increased over the past few decades, and this is largely attributed to increased detection by diagnostic tools (1, 3). RCC is subdivided into various histotypes based on tumour morphology, genetic signature, clinical manifestation, and area of the nephron where the tumour is present. The most common type of RCC is clear-cell (cc) RCC, accounting for 80% of all cases. Other types included papillary, chromophobe and others, which can be classified as non-cc-RCC (4).

In cc-RCC, the lack of functional Von-Hippel Lindau (*VHL*) tumour-suppressor gene prevents the degradation of hypoxia-inducible factor-alpha (HIF α) under normal oxygen conditions. This leads to the inappropriate expression of hypoxia response genes during normoxia, resulting in a condition called pseudohypoxia. The hypoxia response pathways are constitutively active in pseudohypoxia, which causes the resulting cells to be proliferative, invasive, and angiogenic (5). A shift in metabolism, known as the Warburg effect, also occurs, wherein cells have a strong preference for glycolysis over aerobic respiration, even when oxygen is readily available (6). The inactivation of *VHL* and the Warburg effect provide cancerous cells with an environment that further facilitates rapid proliferation.

Hydrogen sulfide (H₂S) is a small, membrane-permeable gasotransmitter that is produced in nearly all mammalian cells by three independent enzymes: Cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST) (7). H₂S modifies cellular protein activity by reacting with cysteine thiol groups in a process called *S*-sulfhydration (8). Endogenous H₂S production is critical for the normal functioning of various systems of the human body, including the central nervous system, cardiovascular system, digestive system, endocrine system, and respiratory system (9).

Under hypoxic conditions, H₂S pushes cells towards glycolysis by increasing glyceraldehyde-3 phosphate dehydrogenase activity. It also promotes anti-apoptotic signalling by *S*-sulfhydration of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF κ B) and modulates angiogenesis by interacting with vascular endothelial growth factor (10). However, when the H₂S level becomes too high under normoxic conditions, it acts as a mitochondrial poison that inhibits complex-IV of the electron transport chain, which prevents aerobic respiration (11). The level of endogenous H₂S production is monitored precisely by the body. Unlike hormones and neurotransmitters, gasotransmitters such as H₂S are not suitable for long-term storage. As soon as H₂S is produced, it is immediately able to react with its surroundings, therefore production must be precisely regulated at the level of enzyme expression and activity (12).

Given its involvement in physiological functions, H₂S production has been linked to various types of cancer including colon, ovarian, breast, liver (13-15). Szabo *et al.* have shown that CBS is up-regulated in colon cancer. They observed increased expression of CBS in both human tumour samples and malignant cell lines when compared to non-malignant samples. Increased endogenous H₂S production due to increased CBS expression and activity contributes to cell migration and bioenergetics (16). Additionally, overexpression of CSE was observed in ovarian cancer cell lines that expressed little to no CBS, unlike ovarian epithelial cells that expressed CBS, suggesting an inverse correlation between

Table I. Patient demographics.

Demographic characteristics	Value	
Number of patients, n	Total	88
Age, years	Mean \pm SD	64.3 \pm 12.35
Gender, n	Male:female	59:29
BMI, kg/m ²	Mean \pm SD	30.7 \pm 7.18
Type of surgery, n	PN:RN	52:36
Specimen laterality, n	L:R	43:45
Follow-up time, months	Mean \pm SD	70 \pm 6.6

PN: Partial nephrectomy; RN: radical nephrectomy; L: left; R: right; SD: standard deviation.

expression of these two H₂S-producing enzymes. Furthermore, the same study found that silencing CBS significantly inhibited ovarian cancer cell proliferation as it reduced H₂S production, activated tumour suppressors (including *p53*), and impaired cytoprotection by inhibiting NF κ B (17).

Data on enzymes responsible for endogenous H₂S production are controversial, showing them to be increased (6) or decreased (6, 18) in RCC cell lines. H₂S levels were observed to be higher in *VHL*-deficient cc-RCC cell lines and inhibition of CBS and CSE significantly reduced the growth, survival, and metabolic output of cc-RCC cell lines (6). However, another study showed H₂S-producing enzymes to be reduced up to 17-fold in cc-RCC cell lines (18).

The aim of this study was therefore to determine whether the expression patterns of H₂S-producing enzymes increased or not in human RCC tissues and whether these correlated with subsequent clinical outcomes. If any correlations between CSE, CBS or MPST expression and patient outcomes emerge, they may support the use of this approach as a potential prognostic tool for patients with RCC.

Patients and Methods

Immunohistochemistry. This study was approved by the University of Western Ontario's Research Ethics Board (REB 106749). Eighty-eight human kidney tissue specimens were collected from storage at the University Hospital – London Health Sciences Centre Pathology Department. These specimens were obtained from patients dating back from 2012 who presented with malignant kidney mass and underwent partial or radical nephrectomy. Each specimen had tumour tissue adjacent to normal, healthy tissue. From each tissue sample, three slices were obtained from the tumour section and three slices from the normal tissue section. Samples were then stained with antibodies for CBS (mouse anti-human CBS; Abnova, Walnut, CA, USA), CSE (rabbit anti-human CSE; Sigma-Aldrich, St. Louis, MO, USA), and MPST (rabbit anti-human MPST; Sigma-Aldrich). The primary antibody for CBS was used at a 250:1 dilution, whereas antibodies for CSE and MPST were used at a 1500:1 dilution, based on preliminary optimization to ensure strong enzyme staining of our tissue samples.

Imaging and analysis. Immunohistochemically stained samples were imaged with a Nikon camera mounted on a bright-field microscope

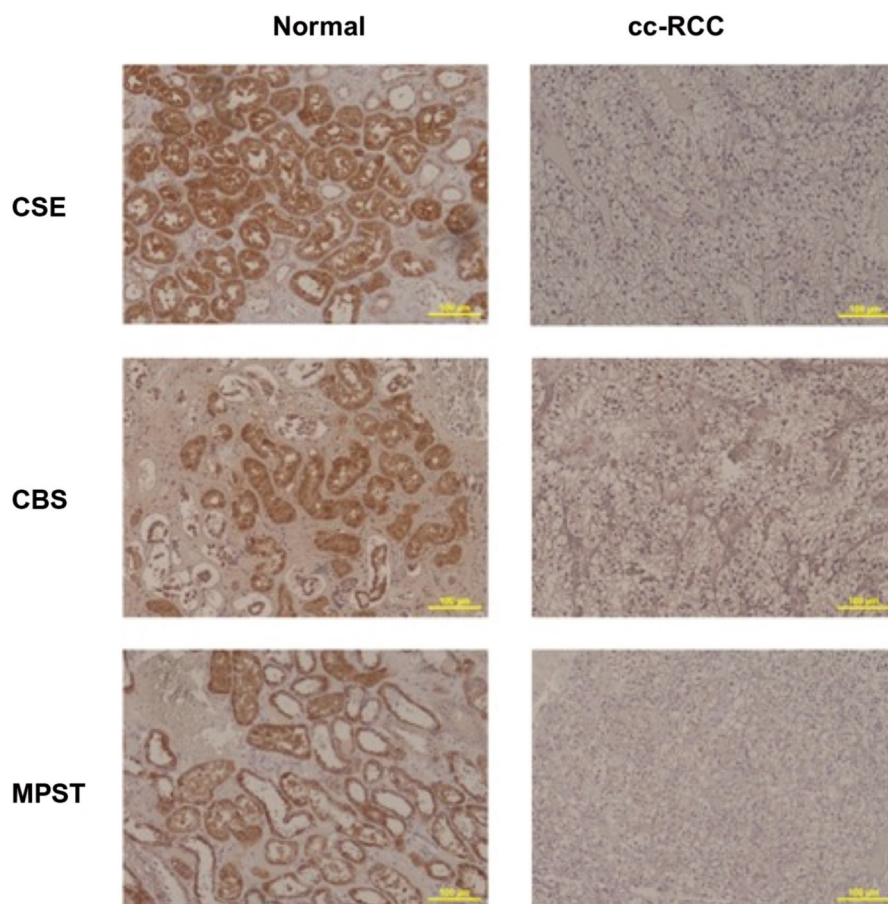


Figure 1. Immunohistochemistry staining of formalin-fixed, paraffin-embedded human kidney tissue. Each specimen consisted of normal tissue adjacent to renal cell carcinoma (RCC) tissue. Each section was immunohistochemically stained with antibodies for cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST). All images were taken at 10 \times magnification using bright-field microscopy.

(Nikon Eclipse 90i; Nikon, Melville NY, USA). Images were taken at 10 \times magnification. Since tumours are heterogeneous, images were taken from three random areas on each slide to account for this.

All images were analyzed using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MA, USA). The colour threshold tool on ImageJ was used to quantify areas positive for enzyme staining to compare expression patterns in cancerous *versus* healthy tissue. The mean pixel intensity (MPI) of the stained areas, which was taken to be representative of the relative enzyme expression in that area of tissue, was used for subsequent data analysis. The MPI for the three tumour and three normal tissue sections were averaged for each specimen. To ensure consistency of analysis, the optimal threshold parameters for each enzyme stain were saved as a template and applied to all images analyzed for that particular enzyme. The area quantified was confirmed with a clinical pathologist to ensure accuracy. Subsequently, in order to determine the fold-increase of the expression of each enzyme in the patient-matched biopsies, the ratio of the MPI of each enzyme stain of RCC relative to that of normal tissue samples was calculated.

GraphPad Prism Version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for data analysis. A paired sample *t*-test was conducted to compare relative enzyme expression between

cancerous and healthy tissue for each enzyme. A one-way repeated measures ANOVA analysis and Tukey's *post hoc* test were conducted to determine statistical differences between ratios of enzyme expression.

Retrospective analysis. Retrospective analysis was performed using PowerChart software (Cerner, Kansas City, MO, USA) to obtain data of patient demographics, nuclear grade, tumour size, metastasis, and recurrence. These data were cross-referenced with immunohistochemistry results to detect any relationships between expression of H₂S-producing enzymes and patient outcomes. Two-way ANOVA analyses and Tukey's *post hoc* tests were conducted to determine statistical significance for each correlation.

Results

Expression of H₂S-producing enzymes increases in RCC versus paired healthy renal tissue. Eighty-eight human kidney samples and demographics were analyzed. Demographics of the patients in this study are shown in Table I.

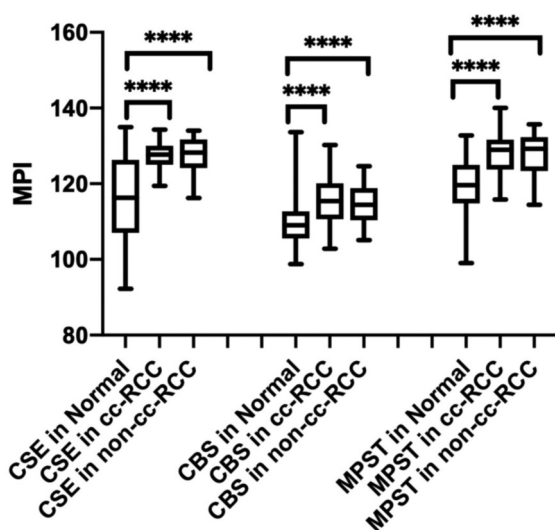


Figure 2. Expression patterns of cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST) in normal (N), clear-cell renal cell carcinoma (cc-RCC) and non-cc-RCC tissue. Box and whisker graph of expression in patient-matched biopsies from healthy (normal, n=88) and cancerous (cc-RCC, n=68; non-cc-RCC, n=20) kidney tissues which were fixed, paraffin-embedded, sectioned, and immunohistochemically stained for CSE, CBS, and MPST. Data are presented as the mean \pm SD of the mean pixel intensity. ****Significantly different at $p < 0.0001$ by two-way ANOVA and Tukey's post hoc tests.

In order to evaluate the expression of H₂S-producing enzymes in RCC compared with normal renal tissue, immunohistochemistry was used to stain three healthy and three tumour sections from each patient for CBS, CSE and MPST expression. Side-by-side sample images of paired normal and cc-RCC sections stained for CSE, CBS and MPST expression are shown in Figure 1.

We found that the MPI of CSE, CBS, and MPST were significantly greater both in cc-RCC and non-cc-RCC tissue versus normal tissue ($p < 0.0001$). There was no significant difference in MPI between cc-RCC and non-cc-RCC for CSE ($p = 0.914$), CBS ($p = 0.662$), and MPST ($p = 0.681$) (Figure 2).

H₂S-producing enzymes exhibit a differential increase in expression. To determine the fold-increase of each enzyme, the ratio of the difference in MPI of CSE, CBS, and MPST between RCC and normal samples, was calculated. The three H₂S-producing enzymes showed differential changes in expression when comparing the ratio of expression in tumour versus normal kidney tissue (Figure 3). There was a 1.10-fold increase in expression of CSE in RCC tissue versus normal, healthy tissue. The ratio for CSE was found to be significantly greater than those for CBS (1.051 ± 0.06 , $p < 0.001$) and MPST (1.083 ± 0.08 , $p = 0.01$). There was no

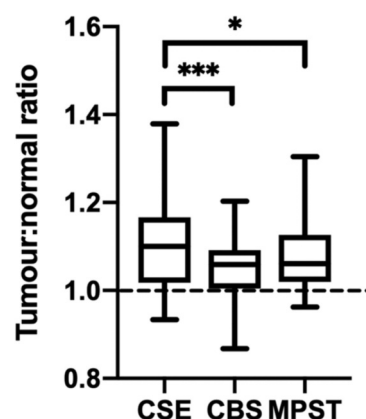


Figure 3. The ratio of tumour:normal tissue expression of cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST) in human kidney tissue. Box and whisker graph showing the mean pixel intensity ratio of tumour:normal tissue with min-max values. The dotted line represents a 1:1 ratio of mean pixel intensity, where there is no difference in enzyme expression between tumour and normal tissue. Significantly different at $*p < 0.01$ and $***p < 0.001$ by one-way repeated measures ANOVA and Tukey's post hoc tests.

significant difference in the MPI ratio between CBS and MPST ($p = 0.65$).

H₂S-producing enzyme expression patterns in RCC specimens do not correlate to grade, pathological stage, recurrence or metastasis. In order to determine whether patterns of endogenous H₂S-producing enzyme expression correlated with subsequent pathological and clinical outcomes, the ratios of enzyme expression of patient-matched biopsies from healthy and RCC kidney tissue were compared between patients whose RCC scored low (1 or 2) or high (3 or 4) on the Fuhrman grading system, had different pathological stages in the pathology report, and whose cancer did or did not recur/metastasize after surgical treatment. There were no significant differences in the MPI ratio for CSE, CBS and MPST based on correlation with grade ($p = 0.061$, $p = 0.95$ and $p = 0.212$), stage ($p = 0.714$, $p = 0.266$ and $p = 0.336$), and metastasis/recurrence ($p = 0.352$, $p = 0.731$ and $p = 0.709$) (Figure 4).

Discussion

The incidence of RCC has been rising by 2% annually over the past two decades due to the increased use of diagnostic tools for other reasons (3). Due to the lack of specific early symptoms and the absence of general screening tests, more than 30% of patients with RCC have disease at the metastatic stage at the time of diagnosis (19). Despite diagnostic advances, RCC is still the most lethal common urological

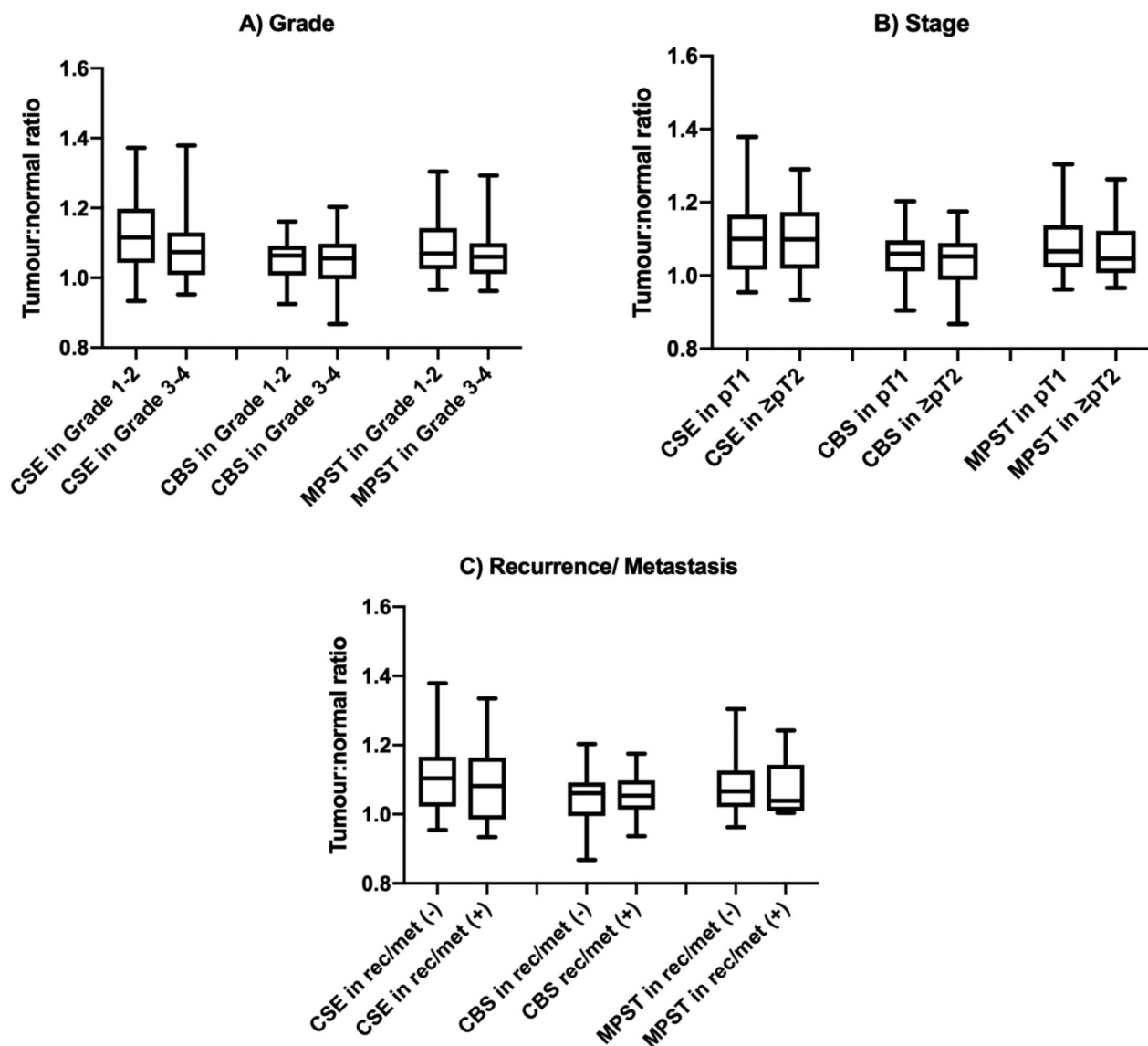


Figure 4. Mean pixel intensity (MPI) ratio of tumour:normal tissue expression of cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST). Box and violin graph represented the mean pixel intensity ratio between patients whose renal cell carcinoma scored low (1 or 2, $n=40$) or high grade (3 or 4, $n=46$) on the Fuhrman grading system (A), with lower ($pT1$, $n=62$) or higher stage ($\geq pT2$, $n=26$) in the pathology report (B), and whose cancer either did (positive, $n=16$) or did not (negative, $n=72$) recur/metastasize in the follow-up period (C). Data are presented as a ratio of mean tumour:normal pixel intensity \pm SD. There were no significant differences between groups by two-way repeated-measures ANOVA and Tukey's post hoc tests.

cancer (1). The recent studies have reported increased H₂S levels and H₂S-producing enzyme expression in several types of urological cancer, including RCC (6, 20-22). Thus, this study aimed to evaluate whether the expression patterns of H₂S-producing enzymes in human RCC samples correlate with subsequent clinical outcomes. The results showed a differential increase in the expression of CSE, CBS, and MPST in both cc and non-cc-RCC tissue samples; however, the expression patterns did not correlate with clinical outcomes.

H₂S has been a focus in cancer research because of its involvement in cytoprotection (23), angiogenesis (13), and anaerobic metabolism (24). In hypoxia, H₂S accumulates and helps maintain cell function by up-regulating glycolysis to provide ATP *via* anaerobic metabolism, up-regulating cytoprotective pathways by promoting anti-apoptotic signaling of NF κ B, and promoting new blood vessel formation to deoxygenated tissue by interacting with vascular endothelial growth factor (13, 25, 26). Since it is a

membrane-permeable gasotransmitter and direct measurement of H₂S in the cell is not easy, we measured H₂S-producing enzyme expression and not the H₂S level directly, the increase in H₂S-producing enzyme expression observed in this study suggests that higher H₂S activity may also be present in human cc and non-cc-RCC samples. Thus, the findings of this study contribute to the literature surrounding the role of H₂S and H₂S-producing enzymes in RCC, using human RCC samples.

Previous studies have examined H₂S-producing enzyme expression profiles in other types of human cancer. Szabo *et al.* showed that CBS was up-regulated but CSE unchanged in human colon cancer, resulting in an increased rate of H₂S production. They further discovered that colon cancer-derived epithelial cell lines also up-regulated CBS and increased H₂S production (16). This indicates that in the colon cancer, CBS is the main regulator in supporting growth, proliferation, and angiogenesis of the cancerous cells. Another study by Bhattacharyya *et al.* demonstrated an inverse correlation between the expression of CBS and CSE in human ovarian cancer. They observed overexpression of CSE in ovarian cell epithelium; however, CBS was overexpressed in ovarian cancer cell lines (17). Unlike these studies, we found a differential increase in the expression of all three H₂S-producing enzymes in the cc and non-cc RCC samples. Additionally, CSE exhibited significantly higher tumour: normal tissue expression than CBS and MPST, suggesting its importance in the pathology of RCC specifically.

The observed increase in the expression of H₂S-producing enzymes suggests that inhibiting these enzymes may represent a novel therapeutic strategy for limiting RCC tumour growth. Previous studies have evaluated the effects of inhibiting key H₂S-producing enzymes in various tumour models. Szabo *et al.* showed that silencing of CBS in colon cancer cells reduced xenograft growth of patient-derived colon cancer xenograft in mice and suppressed neovessel density (16). Bhattacharyya *et al.* showed the silencing of CBS inhibited ovarian cancer cell proliferation by reducing H₂S production, activating tumour suppressors such as p53, and impairing cytoprotection by inhibiting NFκB (17). Based on the anti-tumour effects of inhibiting H₂S-producing enzymes in these studies, it is likely that this approach would also limit RCC tumour growth. Sonke *et al.* have shown that inhibition of CBS and CSE significantly reduced the growth, survival, and metabolic output of murine RCC cells. Furthermore, they also showed that inhibition of endogenous H₂S production in chick embryos also restricts the neovascularization and growth of cc-RCC xenografts (6). Another study by Breza *et al.* showed that silencing of especially of CSE might contribute to resistance of induction of apoptosis (18). Further research is needed to establish whether inhibiting H₂S-producing enzymes, particularly CSE, inhibits the growth of murine RCC tumours *in vivo* and human RCC cell lines.

While we are the first to show that CSE, CBS, and MPST are differentially increased in both cc and non-cc human RCC tissue samples, quantifying enzyme expression is not sufficient to conclude that H₂S production is increased in human RCC. To determine the activity of the H₂S-producing enzymes, H₂S levels need to be measured or functional assays need to be performed using human RCC samples. Additionally, a larger cohort of patient samples is needed to confirm whether the differential increase in CSE, CBS and MPST expression correlates with clinical outcomes. While no correlations were observed in this study, Shackelford *et al.* showed that CBS protein level increased in RCC tumours with higher Fuhrman grades (20). If it is established that H₂S-producing enzyme activity increases in RCC and is correlated with clinical outcomes, healthcare professionals can consider using a nomogram that correlates expression of H₂S-producing enzyme in a renal biopsy with patient demographics to give a calculated score that predicts the likelihood of a mass becoming aggressive, metastasizing, or recurring. This would allow healthcare teams to tailor an individualized treatment strategy and make an informed decision on the direction of oncological treatment.

In conclusion, this is the first study to report the differential increase in the expression of CSE, CBS, and MPST in human RCC. Since these patterns did not correlate with cancer stage, recurrence, metastasis, or nuclear grade in this study, further research is needed to establish whether H₂S-producing enzyme expression might serve as a novel diagnostic/prognostic marker. Additionally, targeted therapies aimed at inhibiting these H₂S-producing enzymes may represent a novel therapeutic approach towards limiting RCC tumour growth and disease progression.

Conflicts of Interest

The Authors declared that they have no conflicts of interest in regard to this study.

Authors' Contributions

S. Emrullah: Data collection or management, data analysis, article writing; P. Katharine: data collection or management, data analysis, article writing; J. Smriti: data analysis, article writing; A. Masoud: article editing; G. Manal: project development; S. Alp: project development, article writing/editing.

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